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| (54) Title: RETRO-, INVERSO-, AND RETRO-INVERSO SYNTHETIC PEPTIDE ANALOGUES (57) Abstract <p>Synthetic peptide antigen analogues of native peptide antigens with partial or complete retro, inverso or retro-inverso modifications are provided. When administered as an immunogen to an immunocompetent host the synthetic peptide antigen analogues induce the production of antibodies which recognise the native peptide antigen. Uses of these analogues, vaccines and methods of preparing vaccines comprising these antigen analogues, and antibodies generated using these antigen analogues are also provided.</p> | | |

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RETRO-, INVERSO-, AND RETRO-INVERSO SYNTHETIC PEPTIDE ANALOGUES

TECHNICAL FIELD

The invention relates to synthetic peptide antigen analogues of native peptide antigens with partial or complete retro, inverso or retro-inverso modifications. When administered as an immunogen to an immunocompetent host the synthetic peptide antigen analogues induce the production of antibodies which recognise the native peptide antigen. The invention also relates to uses of these analogues, to vaccines and methods of preparing vaccines comprising these antigen analogues, and to antibodies generated using these antigen analogues.

BACKGROUND ART

The stereochemistry of polypeptides can be described in terms of the topochemical arrangement of the side chains of the amino acid residues about the polypeptide backbone which is defined by the peptide bonds between the amino acid residues and the α -carbon atoms of the bonded residues. In addition, polypeptide backbones have distinct termini and thus direction.

The majority of naturally occurring amino acids are L-amino acids. Naturally occurring polypeptides are largely comprised of L-amino acids.

D-amino acids are the enantiomers of L-amino acids and form peptides which are herein referred to as inverso peptides, that is, peptides corresponding to native peptides but made up of D-amino acids rather than L-amino acids.

Retro peptides are made up of L-amino acids in which the amino acid residues are assembled in opposite direction to the native peptide sequence.

Retro-inverso modification of naturally occurring polypeptides involves the synthetic assemblage of amino acids with α -carbon stereochemistry opposite to that of the corresponding L-amino acids, i.e. D- or D-allo-amino acids, in reverse order with respect to the native peptide sequence. A retro-inverso analogue thus has

reversed termini and reversed direction of peptide bonds while approximately maintaining the topology of the side chains as in the native peptide sequence.

Partial retro-inverso peptide analogues are polypeptides in which only part of the sequence is reversed and replaced with enantiomeric amino acid residues. Since the retro-inverted portion of such an analogue has reversed amino and carboxyl termini, the amino acid residues flanking the retro-inverted portion are replaced by side-chain-analogous α -substituted geminal-diaminomethanes and malonates, respectively.

Processes for synthesis of retro-inverso peptide analogues (Bonelli et al., 1984; Verdini and Viscomi, 1985) and some processes for the solid-phase synthesis of partial retro-inverso peptide analogues have been described (Pessi et al., 1987).

It has been observed that due to the stereospecificity of enzymes with respect to their substrates, replacement of L-amino acid residues with D-amino acid residues in peptide substrates generally abolishes proteolytic enzyme recognition and/or activity, although exceptions are known.

Peptide hormones have been of particular interest as targets for retro-inversion, presumably because their analogues would have potential use as therapeutic agents. Partial, and in a few cases complete, retro-inverso analogues of a number of peptide hormones have been prepared and tested (see, for example, Goodman and Chorev, 1981).

Complete or extended partial retro-inverso analogues have generally been found to be devoid of biological activity. The lack of biological activity has been attributed to possible complex structural changes caused by extended modification, the presence of reversed chain termini or the presence of proline residues in the sequences. Some partial retro-inverso analogues, that is peptides in which only selected residues were modified, on the other hand, have been shown to retain or enhance

biological activity. Retro-inversion has also found application in the area of rational design of enzyme inhibitors.

5 The fact that retro-inversion of biologically active peptides has met with only limited success in retaining or enhancing the activity of the native peptide is probably due to several reasons. Although structurally very similar, it was realised early that peptides and their retro-enantiomers are topologically not identical
10 and crystal structure and solution conformation studies have borne this out. Biological activity of a peptide hormone or neurotransmitter depends primarily on its dynamic interaction with a receptor, as well as on transduction processes of the peptide-receptor complex.
15 It is now clear that such interactions are complex processes involving multiple conformational and topological properties. Consequently it is not surprising that a retro-inverso analogue may not be able to mimic all of these properties.

20 The development of synthetic peptide vaccines has been a very active field of research over the past two decades (Arnon, 1991; Steward and Howard, 1987). Unfortunately, not much is known about the chemistry of antigen-antibody binding; only very few X-ray crystal
25 structures of antibody-antigen complexes have been solved to date (Davies et al., 1988). As a result, prior to the present invention, it was not possible to predict if antibodies could be elicited against an inverso, retro or retro-inverso peptide and if such antibodies would be
30 capable of recognising the native peptide antigen from which the peptide sequence was derived. Lerner and co-workers (Lerner, 1984) report the synthesis of native, retro-, inverso- and retro-inverso forms of an influenza virus haemagglutinin peptide. They claim that antibodies
35 raised against these peptides are not cross-reactive and that only antibodies against the native form peptide bind to the native peptide antigen.

Oral immunisation, with the production of secretory immunoglobulin A (IgA) antibodies in various mucosae, has been used for many years, particularly for gastrointestinal infections. Successful induction of a systemic immune response to an orally administered polypeptide antigen requires that at least some of the antigen is taken up into the circulation. It is now known that intestinal peptide transport is a major process, with the terminal stages of protein digestion occurring intracellularly after non-specific transport of peptides into the mucosal absorptive cells. There is also irrefutable evidence that small amounts of intact peptides and proteins do enter the circulation from the gut under normal circumstances. Due to inefficient intestinal absorption and due to proteolytic degradation of 'native' polypeptide antigens, the amount of antigen required for oral immunisation generally far exceeds that required for parenteral induction of systemic immunity. Furthermore, oral presentation of such large quantities of antigen often leads to the simultaneous induction of IgA/suppressor T-cell-mediated systemic tolerance which acts to reduce the production of immunoglobulin G (IgG) antibodies. Therefore, a need exists for non-tolerogenic effective oral vaccines which can withstand proteolytic attack.

DISCLOSURE OF THE INVENTION

Definitions

Throughout the specification and claims "retro modified" refers to a peptide which is made up of L-amino acids in which the amino acid residues are assembled in opposite direction to the native peptide with respect to which it is retro modified.

Throughout the specification and claims "inverso modified" refers to a peptide which is made up of D-amino acids in which the amino acid residues are assembled in the same direction as the native peptide with respect to which it is inverso modified.

Throughout the specification and claims "retro-inverso modified" refers to a peptide which is made up of D-amino acids in which the amino acid residues are assembled in the opposite direction to the native peptide with respect to which it is retro-inverso modified.

Throughout the specification and claims the term "native" refers to any sequence of L amino acids used as a starting sequence for the preparation of partial or complete retro, inverso or retro-inverso analogues.

The term "peptide" as used throughout the specification and claims is to be understood to include peptides of any length.

Throughout the specification and claims the term "antigenic fragment" refers to a peptide which is a portion of an antigen which itself is immunogenic or capable of binding antibodies.

The term "antigen" as used throughout the specification and claims is to be understood to include immunogens as the context requires.

Throughout the specification and claims the term "antigen analogue" refers to a peptide molecule capable of mimicking the immunological activity of the native peptide antigen with respect to which it is partially or completely retro, inverso or retro-inverso modified.

Partial modification includes analogues in which as few as two consecutive residues are modified. Typically at least 5 or 6 consecutive residues are modified.

Other amino acids, usually, but not restricted to L isomers, can be added to the antigen peptide for purposes such as conjugation or increasing solubility. Cysteine can be included as its AcM derivative to prevent polymerisation or cyclisation of a peptide or replaced by amino butyric acid.

The present invention relates to partially or completely retro, inverso or retro-inverso modified antigen analogues of native peptide antigens which, when administered to an immunocompetent host as an immunogen, induce the production of antibodies which recognise the

native antigen. Surprisingly, the antigen analogues in accordance with the invention have been shown to have immunological activity and are therefore candidates for the preparation of vaccines. Incorporation of D-amino acids into peptide antigen analogues increases their stability to degradation after administration. Further, incorporation of D-amino acids has potential for oral administration of analogues. Having shown that antibodies can be elicited against retro, inverso and retro-inverso antigen analogues, which are capable of recognising the native peptide antigen from which the sequence of the analogue was derived, it follows that generally retro, inverso and retro-inverso antigen analogues can be expected to be successful since antibody-antigen binding interactions are not fundamentally different from case to case.

According to a first aspect of the present invention, there is provided a synthetic peptide antigen analogue of a native peptide antigen, which analogue is partially or completely retro modified with respect to the native antigen.

According to a second aspect of the present invention there is provided a synthetic peptide antigen analogue of a native peptide antigen, which analogue is partially or completely inverso modified with respect to the native antigen.

According to a third aspect of the present invention, there is provided a synthetic peptide antigen analogue of a native peptide antigen, which analogue is partially or completely retro-inverso modified with respect to the native antigen. The analogues of the invention induce the production of antibodies which recognise the native peptide antigen when administered as an immunogen to an immunocompetent host. In the case of retroinverso analogues it is recognised that further modification can be required in special cases. Where the chosen peptide antigen is smaller than the average size of an antibody-binding antigenic structure, then the C

and N-terminal groups will be as important as the internal residues in recognition of and binding to the antibody. A completely retro-inverso version of such a peptide antigen is likely to differ sufficiently from the native peptide antigen at its ends to render it ineffective as an antigen analogue. For such peptides it is desirable to either produce polymers made up of multiple copies of the peptide or modify the ends of the peptides by protecting them with, for instance, additional residues joined to them or chemically replacing them by side-chain-analogous-substituted geminal diaminomethanes and malonates. Other techniques such as cyclizing these peptides may also be beneficial.

Typically, the antibodies raised are capable of neutralising deleterious biological activity of the native peptide antigens, however, it is to be understood that antibodies raised against the analogues of the invention which are able to bind the native peptide antigen, whether or not they are also capable of such neutralisation are of use, for instance, in diagnostic applications. Given that the antigen analogues of the invention lead to production of antibodies which recognise the native antigen it follows that they are candidates for vaccine components in situations where vaccination against a native antigen is desirable. It is recognised that in some individuals in a population of test animals a portion will fail to respond to immunisation because of major histocompatibility complex (MHC) restriction. However those members of the population which do respond, respond very well. This lack of response is a common immunological phenomenon and should not be considered to be an indication that retroinverso antigen analogues have variable efficacy.

The invention also encompasses the antigen analogues of the invention when used to immunise an immunocompetent host.

According to a fourth aspect of the present invention there is provided a vaccine comprising at least

one antigen analogue of the present invention together with a pharmaceutically or veterinarily acceptable carrier, diluent, excipient and/or adjuvant.

Vaccines of the invention may comprise antigen
5 analogues conjugated with a suitable carrier or synthesised with the analogue and a proteinaceous carrier forming a continuous polypeptide.

Vaccines of the invention can be formulated using standard methods in the art of vaccine formulation.

10 Selection of appropriate diluents, carriers, excipients and/or adjuvants can be made in accordance with standard techniques in the art.

Preferably, those synthetic peptide antigen analogues of the invention containing D-amino acids are
15 capable of eliciting immune responses which last longer than the immune response obtained with the corresponding native antigen.

Typically the native antigen is any naturally occurring polypeptide or antigenic fragment thereof,
20 which is capable of eliciting an immune response in a host. Native antigens in accordance with the invention include peptides or polypeptides of any length whose amino acid sequences stem from polypeptides of pathogens such as poliomyelitis, hepatitis B, foot and mouth
25 disease of livestock, tetanus, pertussis, HIV, cholera, malaria, influenza, rabies or diphtheria causing agents, or toxins such as robustoxin, heat labile toxin of pathogenic *Escherichia coli* strains and Shiga toxin from *Shigella dysenteriae*. Other antigens of interest include
30 Amyloid β protein (Alzheimer's disease) and human chorionic gonadotropin and gonadotropin releasing hormone (contraceptive vaccines).

Preferred analogues of the invention are analogues of the malarial antigen which is the immunodominant
35 epitope of the circumsporozoite coat protein of *P. falciparum* sporozoites or a diphtheria toxin antigen or an HIV-1 antigen, HBV antigen or robustoxin. More

preferably the analogues are retro-inverso forms of these molecules.

Vaccines of the invention may be administered to hosts in need of such treatment by injection. Vaccines
5 incorporating D-amino acid containing analogues may also be administered orally. When the vaccine is to be administered by injection the antigen analogue can be conjugated to an appropriate carrier molecule and injected via conventional methods for example
10 intramuscularly.

The present invention also provides a method of vaccinating a host in need of such treatment which method comprises administering an effective amount of an antigen analogue or vaccine according to the invention to the
15 host.

In a further aspect of the invention, antibodies produced by immunisation of a host with antigen analogues of the invention are provided. These antibodies are useful as agents in the diagnosis, treatment and/or
20 prophylaxis of diseases, as well as drug delivery.

The invention also provides a method of analysis of a sample for antibodies to a native peptide antigen comprising using an antigen analogue of the antigen according to the invention.

25 The invention additionally provides a method of analysis of a sample for the presence of a native peptide antigen comprising using antibodies of the invention which recognise the antigen.

The invention further provides a diagnostic kit
30 comprising at least one antigen analogue or antibody of the invention, together with positive and negative control standards. Where the kit is to be used to detect antibodies to a particular native antigen, the kit will comprise an antigen analogue of the native antigen. The
35 positive standard may be an antibody of the invention raised to that antigen analogue. The negative standard may be any non-cross-reacting antibody. Where the diagnostic kit is to be used for the detection of a

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native antigen, the kit will comprise antibody to an analogue of the native antigen. The analogue of the native antigen may be used as the positive standard. A peptide not recognised by the antibody is used as
5 negative standard.

The invention also provides a method of preparing an antigen analogue, of a native peptide antigen comprising synthesising a partially or completely retro, inverso or retro-inverso analogue of the native peptide antigen.

10 Other amino acids, usually, but not restricted to L isomers, can be added to the antigen peptide for purposes such as conjugation or increasing solubility. Cysteine can be included as its AcM derivative to prevent polymerisation or cyclisation of a peptide or replaced by
15 amino butyric acid.

The invention further provides a method of preparing a vaccine against a native peptide antigen which method comprises: providing a retro, inverso or retro-inverso analogue of the native peptide antigen; and admixing an
20 effective amount of the antigen analogue with a pharmaceutically or veterinarily acceptable carrier, diluent excipient and/or adjuvant. The method of preparing a vaccine may additionally comprise conjugating the antigen analogue to a suitable carrier molecule.

25 ABBREVIATIONS

| | |
|--------|---|
| Ab | Antibody |
| BOP | (benzotriazolyloxy)tris(dimethylamino) phosphonium hexafluorophosphate (Castro's reagent) |
| DMF | dimethyl formamide |
| 30 BSA | bovine serum albumin |
| ELISA | enzyme-linked immunosorbent assay |
| Fmoc | 9-fluorenylmethoxycarbonyl |
| HPLC | high-performance liquid chromatography |
| Ig | immunoglobulin |
| 35 in | inverso |
| i.p. | intraperitoneal |
| KLH | keyhole limpet haemocyanin |

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- Mod model
no normal (native)
PBS phosphate buffered saline (10 mM phosphate, 150mM NaCl, pH 7.4)
5 Pfp pentafluorophenyl
PVC polyvinylchloride
re retro
ri retro-inverso
TFA trifluoroacetic acid
- 10 Amino Acids:
L-amino acids are indicated by an upper case followed by lower case lettering e.g. Ala indicates L-alanine.
D-amino acids are indicated by all lower case
15 abbreviations, e.g. ala indicates D-alanine.

BRIEF DESCRIPTION OF THE FIGURES

- Figure 1 illustrates modifications which can be made to an amino acid sequence in accordance with the present invention. R^1 , R^2 , R^3 and R^4 represent amino acid
20 side chains, and Xaa represents any amino acid residue.
- Figure 2 shows the results of ELISA of retro-inverso model (mod) peptide-KLH antiserum against different immobilised peptide-BSA. - noMod (\square), riMod (+), reMod (\diamond), inMod (Δ), control 1 (x), control 2 (∇).
25 Figure 3 shows the results of ELISA of malaria (Mal)-peptide antisera (oral immunisation) against peptide-BSA. - Serum/immobilised antigen: riMal/riMal (Δ), riMal/noMal (+), noMal/noMal (\square), noMal/riMal (\diamond), non-immune serum/noMal (X).
- 30 Figure 4 shows the results of ELISA of diphtheria (DIP) peptide-KLH antisera against immobilised peptide-BSA. - Serum/immobilized antigen: riDip/riDip (Δ), riDip/noDip (+), noDip/noDip (\square), noDip/riDip (\diamond), non-immune/noDip (x).
- 35 Figure 5 shows the results of ELISA of diphtheria (Dip) peptide-KLH antisera against immobilised diphtheria

toxin. - Anti-riDip (+), anti-noDip (□), non-immune serum (◇).

Figure 6 shows the results of ELISA of orally induced diphtheria (Dip) peptide antisera against immobilised diphtheria toxin. - Anti-noDip, 2 weeks (□); anti-riDip, 2 weeks (+), anti-riDip, 8 weeks (◇).

Figure 7 shows the results of ELISA of HIVgp41(735-753) antisera against immobilized peptide: serum/immobilised antigen.

Figure 8 shows the results of ELISA of HIVgp41 monoclonals with gp41 peptides.

Figure 9 shows graphically the ELISA results generated in Example 14.

□ riHBV plate coating
■ noHBV plate coating

Figure 10 shows graphically the ELISA results generated in Example 15.

S1: no-peptide-coated wells
S2: ri-peptide-coated wells.

Figure 11 shows graphically the ELISA results generated in Example 16.

Bold lines: Antisera to ri-HBV peptide
Normal lines: Antisera to no-HBV peptide
Circle and diamond symbols: no-HBV peptide-coated wells.
Square and triangle symbols: ri-HBV peptide-coated wells.

Figure 12 shows graphically the ELISA results generated in Example 17.

Bold lines: Antisera to ri-HBV peptide
Normal lines: Antisera to no-HBV peptide
Circle and diamond symbols: no-HBV peptide-coated wells.
Square and triangle symbols: ri-HBV peptide-coated wells.

BEST MODE OF CARRYING OUT THE INVENTION

Antigen analogues of the invention are prepared by standard techniques for the preparation of L and D amino acid containing peptides, particularly as outlined in
5 Example 1.

Vaccines of the invention are formulated by standard techniques for vaccine formulation using standard carriers, diluents, excipients and/or adjuvants suitable for the formulation of oral or injectable
10 vaccines. Effective amounts of antigen analogues to be incorporated in the vaccines can be determined in accordance with standard methods.

The vaccination regimes used are standard regimes for the vaccination of animal or human hosts. These
15 regimes can be used where immunisation of the host is desired or where the host is being used to produce antibodies for exogenous use. Diagnostic kits of the invention are prepared by standard methods for the preparation of the reagents and controls of such kits.

20 Generally the diagnostic kits are in radio immunoassay (RIA) or immunofluorescence or ELISA formats: the latter can be carried out as described in Example 4.

Where the analogues or antibodies raised against them are used in the detection of a native antigen or an
25 antibody against it, the appropriate agent can be used in immunoassay format against a sample to be tested with appropriate controls.

The invention is further described in the following examples which are illustrative of the
30 invention but in no way limiting on its scope.

The following examples show that antigen analogues in accordance with the invention surprisingly can elicit antibodies capable of recognising the native sequence,
not only in the form of a peptide, but also when
35 contained in the protein from which it was derived.

Three antigen sequences were chosen and tested for their ability to produce antibodies capable of recognizing and interacting with the parent native sequence (Examples 5,

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6 and 7). One antigen sequence (Example 5) was a model without biological relevance, while the other two antigen sequences (Examples 6 and 7) represent synthetic peptide antigens whose potential usefulness as vaccines against malaria and diphtheria, respectively, has been demonstrated previously. The observed cross-reactivity of antibodies against 'native' and retro-inverso peptides strongly indicates that antibodies recognise antigens primarily by the constellation of their amino acid side chains independently of their backbone. In all three cases studied polyclonal antibody preparations against retro-inverso peptide antigens appeared to bind equally well to the parent sequence and to the retro-inverted antigen.

Conventional immunisation with retro-inverso antigens conjugated to carrier proteins, and oral immunisation with free retro-inverso antigens, are shown to be feasible. The ability to induce cross-reactive serum antibodies to retro-inverso antigens indicates the utility of such antigens as vaccines. As demonstrated, these antigens are amenable to immunisation without carrier proteins, not only by injection, but also by oral administration. Probably this is dependent on at least one T-cell epitope being present. This observation, together with the finding that the immune response to retro-inverso antigens is relatively long-lived, indicate the utility of these analogues in overcoming the two main problems of existing experimental synthetic peptide vaccines.

30

EXAMPLE 1

Peptide Synthesis

Peptides were synthesised by a solid-phase method on polyamide (Arshady et al., 1981) or Polyhipe supports using side-chain protected Fmoc amino acids (Carpino & Han, 1972), essentially as described by Eberle et al. (1986). Only pure amino acid derivatives, obtained commercially or by synthesis, were used. The polyamide

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synthesis resins, derivatised with p-alkoxybenzyl alcohol-based linkage agents, were esterified quantitatively with the appropriate preformed C-terminal Fmoc-amino acid symmetrical anhydrides, in the presence
5 of 0.2 molar equivalents of N,N-dimethylaminopyridine and N-methylmorpholine. The Polyhipe resin derivatised with Fmoc-Rink linker (Rink, 1987) did not require esterification of the first amino acid to it. Chain elongation was carried out using Fmoc-amino acid
10 pentafluorophenyl esters (Atherton *et al.*, 1988) or Castro's reagent/1-hydroxybenzotriazole coupling (Hudson, 1988). The progress of each synthesis was monitored using a specific colour test (Hancock & Battersby, 1976) and/or amino acid analysis of acid-hydrolysed peptidyl
15 resin samples.

The peptides were cleaved from the resins and side-chain deprotected with the aid of TFA, containing a suitable mixture of scavenger chemicals (Tam, 1988). After filtration and vacuum evaporation, the peptides
20 were triturated with diethyl ether, collected by centrifugation and lyophilised from aqueous ammonium bicarbonate solution.

All peptides then underwent an initial desalting and purification step by column chromatography on
25 suitable gel filtration media in aqueous solvents. Afterwards they were purified to homogeneity by reversed-phase HPLC using water-acetonitrile (containing 0.05-0.1% TFA) gradient elution. The purity of the synthetic peptides was further assessed by gas-phase acid
30 hydrolysis/amino acid analysis (Bidleymeyer *et al.*, 1987) and, if deemed necessary, by automated gas-phase sequencing (Hunkapiller & Hood, 1983).

EXAMPLE 2Peptide-carrier protein conjugation

Synthetic peptides were coupled via their cysteine thiol groups to carrier proteins using a method adapted from Liu et al. (1979), as follows:

KLH (40mg) or BSA (100mg) was dissolved in 3mL 50mM phosphate buffer, pH 6.0.
m-Maleimidobenzoyl-N-hydroxysuccinimide ester (MBS; 0.2 mL of a fresh 25mg/mL stock solution in N,N-dimethylformamide) was added slowly and the mixture stirred at room temperature for 30 min. It was then immediately filtered through a 0.8mm membrane and pumped onto a column (11 x 170mm) of Sephacryl S-300. This was developed at 0.25mL/min with pH 6 buffer. The protein-MB-containing fractions were collected, pooled and added to prepared peptide solution (10 μ mol in pH 7.5 phosphate buffer). The pH of this mixture was adjusted to 7.5 with 0.1M NaOH; it was then flushed with nitrogen, sealed and stirred magnetically for 2.5h. After exhaustive dialysis (M_r 12,000-15,000 cut-off) against dilute ammonium bicarbonate, the protein-carrier protein conjugates were isolated by lyophilisation. All aqueous solution were degassed prior to use.

EXAMPLE 3Immunisation

Young Swiss albino mice were used for immunisation. Intraperitoneal (i.p.) injections of peptide-KLH conjugates were made without any adjuvant. Oral immunisations were carried out by feeding starved mice with food pellets into which the appropriate antigen solution had been soaked.

EXAMPLE 4ELISA procedures

The wells of PVC microtitre plates were coated with the appropriate antigen (0.25-10 μ g/well), dissolved in dilute carbonate/bicarbonate buffer, pH 9.2, overnight

at 4°C. After aspiration, blocking was effected by incubation for 2h with 4% BSA or boiled casein, 0.05% Tween-20 in PBS. The wells were then washed several times with 0.1% Tween-20 in PBS before addition of
5 antiserum, serially diluted in blocking solution. After incubation for 4h, the plates were again washed. Bound IgG was detected by incubation for 1h with suitably diluted (in blocking solution) affinity-purified anti-mouse IgG-horseradish peroxidase, washing and
10 development with 0.5 mg/mL o-phenylenediamine, 0.01% peroxide at pH 5 in the dark. Colour development was stopped by the addition of 4M sulphuric acid. The plates were then read immediately at a wavelength of 492 nm.

All incubation steps, with the exception of
15 coating, were carried out at room temperature and with agitation of the plates.

EXAMPLE 5

Analysis of antibody production and activity of
antibodies raised to a synthetic antigen sequence without
20 biological relevance

The following model dodecapeptides were synthesised using cysteine thiol protection as the trityl thioether and cleavage of peptidyl resin with 5% thiophenol in TFA for 90 min. Protein conjugates were
25 prepared for the purposes of immunisation.

Normal (native) peptide (L-amino acids, N->C direction) noMod: H-Gly-Cys-Gly-Pro-Leu-Ala-Gln-Pro-Leu-Ala-Gln-Gly-OH (SEQ ID No.1)
Retro-inverso peptide (D-amino acids, C->N direction)
30 riMod: H-Gly-gln-ala-leu-pro-gln-ala-leu-pro-Gly-Cys-Gly-OH

Retro peptide (L-amino acids, C->N direction)
remod: H-Gly-Gln-Ala-Leu-Pro-Gln-Ala-Leu-Pro-Gly-Cys-Gly-OH (SEQ ID No.2)

35 Inverso peptide (D-amino acids, N->C direction)
inMod: H-Gly-Cys-Gly-pro-leu-ala-gln-pro-leu-ala-gln-Gly-OH

Intraperitoneal Immunisation

Groups of four mice per antigen were immunised intraperitoneally with 0.2 mg/dose of KLH-peptide in 0.1mL PBS. Boosters were given 4, 9 and 16 days later.

5 Anti-peptide antibodies in the pooled sera (from each group) were measured in an ELISA using immobilised BSA-peptide conjugates.

It was found that all four antigens, including the D-amino acid-containing ones, were immunogenic. The
10 antisera exhibited very similar antibody levels when assayed against approximately equal amounts of immobilised BSA conjugates of their respective peptide antigens. Furthermore, antibodies in each of the antisera also bound to the other three peptide antigens.
15 The results of one such assay, where binding of the retro-inverso peptide-KLH antiserum to all four peptides was measured, is shown in Figure 2. The fact that not only cross-reaction of noMod and riMod antibodies was observed, but also of antibodies against the
20 non-isosteric antigens, was particularly surprising. This would indicate that the antibodies studied recognise amino acid side chain groupings in the antigen without any regard to the sequence direction and absolute configuration of the α -carbon atoms. Furthermore, the
25 presence of proline residues in the peptide sequences did not prevent the D-amino acid-containing peptides from successfully mimicking the native sequence, as has been observed in retro-inverso peptide hormone analogues.

In order to account for the antibodies specific
30 for the carrier protein attachment site -Gly-Cys-Gly-, i.e. the portion common to all antigens, an antiserum against a KLH conjugate of a 14-residue peptide with completely unrelated sequence, but containing the same attachment site, was also assayed (control 1 in Figure
35 2). As can be seen, antibodies to this site do not appear to contribute significantly to the observed cross-reactivity (control 2 refers to observed binding of the riMod-KLH antiserum to BSA only).

Oral Immunisation

Oral immunisation with the free, i.e. non-conjugated, native and retro-inverso model peptides was carried out as follows: 0.3 mg/dose of the peptides in 50mL PBS was administered to groups of mice according to the schedule outlined above for the i.p. immunisation. The results of the ELISA of the resulting sera are summarised in Table 1. Results from the corresponding immunisations by injection are included for the purpose of comparison.

Table 1
ELISA of model peptide antisera

| | Immobilised Material | Admini- stration route | Antiserum | Days after last booster | Titre ^a |
|----|-------------------------|------------------------------|----------------------|-------------------------------|--------------------|
| 5 | riMod-BSA | i.p. | riMod-KLH | 6 | >5,000 |
| | riMod-BSA | i.p. | noMod-KLH | 6 | >5,000 |
| | riMod-BSA | | non-immune | | 80 |
| 10 | riMod-BSA | i.p. | control ^b | 6 | 64 |
| | BSA only | i.p. | riMod-KLH | 6 | 120 |
| | riMod-BSA | oral | riMod | 6 | 512 |
| | riMod-BSA | oral | riMod | 13 | 2,048 |
| 15 | riMod-BSA | oral | riMod | 26 | 2,048 |
| | riMod-BSA | oral | riMod | 40 | 1,024 |
| | noMod-BSA | i.p. | noMod-KLH | 6 | >5,000 |
| | noMod-BSA | i.p. | riMod-KLH | 6 | >5,000 |
| 20 | noMod-BSA | | non-immune | | 80 |
| | BSA only | i.p. | noMod-KLH | 6 | 64 |
| | noMod-BSA | oral | riMod | 13 | 2,048 |
| | noMod-BSA | oral | noMod | 6 | 0 |
| 25 | noMod-BSA | oral | noMod | 13 | 0 |

a Reciprocal of highest serum dilution giving statistically significant signal.

b Control peptide of 14 residues with unrelated sequence, apart from -Gly-Cys-Gly- protein conjugation site.

Oral immunisation only gave a detectable serum IgG response in the case of the retro-inverso peptide. Again antibodies in the retro-inverso antiserum appeared to cross-react fully with the native peptide. Evidently the retro-inverso peptide, in contrast to the native peptide, was capable of entering the circulation in a sufficient

quantity to mount an IgG response. That the retro-inverso peptide is only slowly degraded is also indicated by the fact that anti-peptide IgG persisted in the blood stream of the animals for many weeks.

5

EXAMPLE 6

a) Analysis of antibody production and activity of antibodies raised to peptide sequences corresponding to the immunodominant epitope of the circumsporozoite coat protein of *P. falciparum* sporozoites

10 The following four peptides, based on the immunodominant epitope of the circumsporozoite coat protein of *P. falciparum* sporozoites, were synthesised:

noMalCys: H-Cys-Asn-Ala-Asn-Pro-Asn-Ala-Asn-Pro-Asn-Ala-Asn-Pro-OH (SEQ ID No.3)

15 noMal: H-Asn-Ala-Asn-Pro-Asn-Ala-Asn-Pro-Asn-Ala-Asn-Pro-OH (SEQ ID No.4)

riMalCys: H-pro-asn-ala-asn-pro-asn-ala-asn-pro-asn-ala-asn-Cys-OH

riMal: H-pro-asn-ala-asn-pro-asn-ala-asn-pro-asn-ala-asn-OH
20

The cysteine derivatives were protected as trityl thioethers for synthesis. Sequence-internal asparagines were coupled without side-chain protection as pentafluorophenyl esters. The deprotection of Fmoc-Asn-Pro-peptidyl resins was shortened to 3 min,
25 followed by three 15 sec DMF washes and immediate acylation with Fmoc-Ala-OH/BOP-HOBt, in order to minimise loss (ca. 50%) of peptide due to diketopiperazine formation. Asparagine was coupled to synthesis resin as the Fmoc-Asn(Mbh)-OH symmetrical anhydride derivative.
30

Cleavage/deprotection was achieved with 5% thiophenol in TFA for the noMalCys- and riMalCys-peptidyl resins and with 5% aqueous TFA for the noMal- and riMal-resins. After cleavage, the crude riMal peptide was further
35 treated for 2h at room temperature with 5% thioanisole in

TFA to remove completely the dimethoxybenzhydryl protecting group.

Groups of mice were immunised i.p. at weekly intervals for a total of four weeks with 100 μ g doses of noMalCys- and riMalCys-KLH conjugates. Blood was collected after five weeks and the serum titres established by ELISA (Table 2).

Table 2

| ELISA of antisera to malaria peptide-KLH conjugates | | |
|---|---------------------|---------------------------------|
| 10 | Immobilised antigen | Antiserum to Titre ^a |
| | noMalCys-BSA | noMalCys-KLH >12,000 |
| | noMalCys-BSA | riMalCys-KLH 1,500 |
| | riMalCys-BSA | noMalCys-KLH >12,000 |
| 15 | riMalCys-BSA | riMalCys-KLH 3,000 |

^a Reciprocal of highest serum dilution showing significant above background reading (average over four individual sera).

20 Here the native antigen was found to be more immunogenic than the retro-inverso antigen. As in the previous examples, the antisera were fully cross-reactive. Mice were immunised orally with the noMal and riMal peptides at weekly intervals for a total of four weeks. Blood was collected and assayed by ELISA after five weeks. The results of the titration are shown in Figure 3. Only the retro-inverso peptide induced a significant titre of anti-peptide antibodies. There appeared to be full cross-reactivity of these antibodies with the normal peptide, in the same way as the peptide-KLH antisera were fully cross-reactive.

b) Testing of no/riMal peptides with sera from malaria patients

35 It has been shown that high levels of antibodies directed against circumsporozoite protein

repeat sequences can inhibit sporozoite development (Mazier et al., 1986). Furthermore, a recombinant vaccine containing such repeats, as well as a peptide vaccine consisting of three NANP repeats coupled to
5 tetanus toxoid, have shown some promise in clinical trials (Herrington et al., 1987 & 1990). After having demonstrated that antisera raised in animals against the noMal and riMal peptides were cross-reactive, it was important to show that anti-sporozoite antibodies in
10 humans with malaria recognised both peptides.

Serum samples from Thai malaria patients were obtained. These sera were known to contain antibodies against the immunodominant epitope of the *Plasmodium falciparum* circumsporozoite protein (Wirtz et al., 1989).
15 With a few exceptions, all the patients had clinically diagnosed malaria and had suffered attacks recently.

We tested these sera for antibodies capable of binding to a recombinant circumsporozoite construct, a synthetic polymer and noMal, all of which contain NANP
20 repeats, as well as riMal. The results are summarised in Table 3. As can be seen, for most sera there is good correlation of the results, the binding to those antigens containing a large number of NANP repeats being stronger. In every case cross-reaction of the antisera between the
25 normal and retro-inverso forms of the (NANP)₃ peptide was observed.

Table 3

IgG antibodies in human sera from malaria sufferers
recognise noMal and riMal peptides

30 Coated ELISA plates were incubated with serially diluted human sera. The plates were then washed and bound antibodies detected using anti-human IgG coupled to horseradish peroxidase. The results were expressed on a scale where + signifies a titre (highest serum dilution
35 giving a significant signal) of 1/320 to +++++ which indicates a titre > 2,560. All results were corrected for non-specific binding by using two non-related

peptides, conjugated to BSA in the same way as noMal and riMal, as controls.

| Immobilised antigen ^a | | | | |
|----------------------------------|-----------------------------------|--------------------------|-----------|-----------|
| Serum | (NANP) ^b ₅₀ | Falc 2.3 CS ^c | noMal-BSA | riMal-BSA |
| 014 | +++ | ++++ | +++ | +++ |
| 015 | ++ | ++ | ++ | + |
| 016 | +++ | +++ | +++ | ++ |
| 5 021 | + | ++ | + | + |
| 022 | ++++ | +++++ | + | ++ |
| 034 | ++ | +++ | + | + |
| 048 | + | +++ | + | + |
| 054 | +++ | ++++ | +++ | ++ |
| 10 055 | ++++ | +++ | +++ | +++ |
| 061 | ++ | ++++ | + | ++ |
| 063 | +++ | +++ | + | ++ |
| 067 | ++ | +++ | + | ++ |
| 119 | ++++ | +++++ | ++++ | +++ |
| 15 121 | ++ | +++ | + | + |
| 122 | +++ | +++++ | ++ | +++ |
| 134 | +++ | ++++ | + | ++ |
| 136 | +++ | ++++ | ++ | ++ |

a ELISA microtitre plates were coated with 10
20 µg/well of the appropriate antigen

b A synthetic polymer, i.e. approximately 50
repeats of the Asn-Ala-Asn-Pro sequence,
prepared according to Etlinger et al. (1988).

c A recombinant protein expressed in yeast (Barr
25 et al., 1987) and containing residues 43 - 391
(Dame et al., 1984) of the *P. falciparum*
circumsporozoite protein.

EXAMPLE 7

Analysis of antibody production and activity of antibodies raised to peptide sequences corresponding to the amino acid sequence of diphtheria toxin

5 The following two peptides, based on the loop sequence of the 14 amino acid residues subtended by the disulphide bridge near the amino-terminus of the diphtheria toxin molecule, were synthesised:

noDip: H-Gly-Asn-Arg-Val-Arg-Arg-Ser-Val-Gly-Ser-Ser-
10 Leu-Lys-Cys-OH (SEQ ID No.5)
riDip: H-cys-lys-leu-ser-ser-Gly-val-ser-arg-arg-val-
 arg-asn-Gly-OH

15 The side-chain protecting groups used were: trityl for cysteine, t-butoxycarbonyl for lysine, t-butyl for serine and 4-methoxy-2,3,6-trimethylbenzenesulphonyl for arginine. Asparagine was coupled as the free side-chain amide and in the form of the pentafluorophenyl ester (Gausepohl et al., 1989) in both cases. Cleavage and
20 the peptidyl resins for 75 min at 0°C with 1M trimethylsilyl bromide-thioanisole in TFA containing 0.25M 1,2-ethanedithiol (Yajima et al., 1988).

25 Mice (4 per antigen) were immunised i.p. with 100µg doses of KLH-conjugated normal and retro-inverso peptide on day 0, followed by boosters on days 4, 9 and 16. Blood was collected on day 21 and the sera pooled. IgG antibodies against the peptides were measured using an ELISA with immobilised BSA-peptide conjugates. The results are summarised in Figure 4. Both the normal and
30 the retro-inverso peptide-KLH conjugates produced similar titres of antibodies, again showing that the presence of unnatural amino acids in the antigen leads to retention of immunogenicity. Furthermore, the results show that IgG in both antisera recognise both antigens equally
35 well.

The antisera were further assayed for their capacity to recognise their antigen sequence in diphtheria toxin itself. An ELISA with immobilised diphtheria toxin (1 μ g/well) was used for this purpose. The results in
5 Figure 5 demonstrate that IgG antibodies in both antisera bound to the toxin. The retro-inverso-peptide-KLH antiserum exhibited a very high antitoxin titre (ca. ten times higher than the titre of the normal antiserum).

A further surprising result was recorded upon oral
10 immunisation with the free native and retro-inverso diphtheria peptides. Here mice were given a single dose of ca. 1mg peptide. After one and two weeks, serum samples from animals immunised with either peptide contained antibodies which bound to diphtheria toxin. In
15 both cases the anti-toxin titre was considerable. Eight weeks after immunisation, on the other hand, only the animals having received the retro-inverso peptide appeared to contain anti-toxin antibodies (Figure 6). Furthermore, the titre measured was very high and
20 comparable to that obtained by boosted i.p. immunisation with the corresponding KLH-conjugate.

EXAMPLE 8

HIV-1 peptides

Many AIDS vaccines consisting of recombinant viral
25 proteins or synthetic peptides have been proposed and are being tested (Spalding, 1992). A variety of epitopes, mostly from the virus coat glycoproteins gp120 and gp41, have been associated with the ability to induce virus-neutralising antibodies. For our studies we chose the
30 gp41 sequences 735-753 (Chanh et al., 1986) and 583-599 (Klasse et al., 1988) and the following peptides were prepared:

noHIVgp41(735-753) H-Tyr-Asp-Arg-Pro-Glu-Gly-Ile-Glu-
Glu-Glu-Gly-Gly-Glu-Arg-Asp-Arg-Asp-
35 Arg-Ser-NH₂ (SEQ ID No.6)

- riHIVgp41 (735-753) H-ser-arg-asp-arg-asp-arg-glu-Gly-
Gly-glu-glu-glu-ile-Gly-glu-pro-arg-
asp-tyr-NH₂
- 5 noHIVgp41 (583-599) H-Leu-Gln-Ala-Arg-Ile-Leu-Ala-Val-
Glu-Arg-Tyr-Leu-Lys-Asp-Gln-Gln-Leu-
NH₂ (SEQ ID No.7)
- inHIVgp41 (583-599) H-leu-gln-ala-arg-ile-leu-ala-val-
glu-arg-tyr-leu-lys-asp-gln-gln-leu-
NH₂
- 10 riHIVgp41 (583-599) H-leu-gln-gln-asp-lys-leu-tyr-arg-
glu-val-ala-leu-ile-arg-ala-gln-leu-
NH₂

The syntheses were carried out as usual but using
synthesis resin with trialkoxydiphenyl-amide linkage
15 (Rink, 1987). Conjugates with BSA and KLH were prepared
by glutaraldehyde polymerisation according to Mariani et
al. (1987). Groups of mice were immunised with 100
 μ g/dose of the HIVgp41(735-753) peptide-KLH conjugates in
Freund's complete adjuvant, followed by two booster
20 injections with Freund's incomplete adjuvant at
fortnightly intervals. Blood was collected two weeks
after the last booster and sera were prepared.

Murine monoclonal hybridoma cell lines producing
antibodies against HIV-1gp41 protein were obtained by
25 immunisation with recombinant gp41. The spleen cells of
the animal with the best immune response were isolated
and fused with NS-1 myeloma cells using polyethylene
glycol-4,000. The resulting cells were plated out and
submitted to limiting dilution cloning.

30 Polyclonal antisera against both the HIVgp41(735-753)
peptides were analysed by ELISA. The results are shown
in Figure 7. ELISA plates were coated with
noHIVgp41(735-753)-BSA and riHIVgp41(735-753)-BSA at 5
 μ g/well for 1 h in carbonate buffer, pH 9.6. After 1 h,
35 the plates were blocked with boiled casein solution.
They were then incubated for 1 h with serially diluted
sera from mice immunised with the peptide-KLH conjugates.
A goat-anti-mouse IgG antibody labelled with horseradish

peroxidase was used for detection. The results were corrected for non-specific binding (determined using unrelated peptide-BSA conjugates). The averages of five data points (corresponding to five individual mice per antigen group) are plotted. The data point labels (e.g. no/no) refer to coated antigen/antiserum. Clearly the extent of cross-reaction between the antibodies to the two forms of the peptide is high. In order to demonstrate that individual antibodies are capable of recognising different forms of a peptide, ELISA experiments were carried out using monoclonal antibodies against gp41. The results with the HIVgp41(583-599) peptides are shown in Figure 8. A library of cell lines producing antibodies against HIVgp41 was prescreened by ELISA using noHIVgp41(583-599)-BSA immobilised on microtitre plates. Twenty four clones were further tested: 100 μ L of cell supernatant was incubated with 5 μ g/well of each of the three peptide-BSA conjugates. The plates were washed and bound antibodies detected with the aid of an anti-mouse IgG-horseradish peroxidase conjugate. The uncorrected colour signals after development with o-phenylenediamine/peroxide are plotted against the cell line designations. In every case where a monoclonal antibody recognised one form of the peptide, it also bound to the other two forms; indeed the extent of cross-reaction appeared to be complete. Some cell lines against gp41 which recognised the HIVgp41(735-753) peptides were also identified, again cross-reaction was found.

30

EXAMPLE 9

Retro-inverso robustoxin (riRtx)

The potentially lethal robustoxin is found in the venom of the male funnel-web spider (Nicholson et al., 1991). It has been shown that immunisation with a vaccine prepared from a synthetic analogue of this toxin can protect mice and monkeys from challenges with native toxin (Mylecharane et al., 1991). In order to

- 30 -

demonstrate the utility of retro-inverso peptides as vaccines, a retro-inverso analogue of robustoxin, having the following sequence, was prepared:

H-cys(Acm)-lys-lys-phe-leu-Gly-thr-ile-thr-thr-glu-
5 cys(Acm)-ser-Gly-gln-gln-asn-tyr-trp-ala-tyr-ile-
cys(Acm)-lys-met-pro-cys(Acm)-cys(Acm)-cys(Acm)-asp-glu-
asn-lys-Gly-cys(Acm)-trp-asn-arg-lys-lys-ala-cys(Acm)-OH

The peptide was purified and its structure verified by gas-phase sequence analysis. The vaccine was prepared by
10 polymerising 0.2 mg of the peptide and 0.8 mg KLH in 1 mL of phosphate-buffered saline with 10 μ L of 25 % aq. glutaraldehyde at room temperature overnight. The reaction was stopped by the addition of 100 μ L of 2 % lysine solution.

15 Groups of mice were immunised with this material or with a vaccine prepared similarly but using normal Cys(Acm)-protected synthetic robustoxin according to the following schedule: fortnightly injections of 50 μ g peptide-KLH, the first two subcutaneously and three more
20 boosters intraperitoneally. These animals, as well as non-immunised controls, were then challenged 14 weeks after the last booster immunisation with 2 minimal lethal doses (the lethal dose determination was carried out immediately prior to the challenge) of funnel-web spider
25 venom by intravenous injection. The non-immunised animals succumbed within ten minutes, whereas those immunised with the native vaccine survived. Of the ri-Rtx-KLH immunised individuals, one expired after 24 h while the remaining seven survived the challenge.

30

EXAMPLE 10

Hepatitis C virus envelope peptide

The following two peptides, based on the envelope sequence (306-330) of the hepatitis C virus, were synthesised.

noCEnv H-Cys-Ser-Ile-Tyr-Pro-Gly-His-Ile-Thr-Gly-
 His-Arg-Met-Ala-Trp-Asp-Met-Met-Met-Asn-Trp-
 Ser-Pro-Thr-Ala-OH (SEQ ID No.8)

riCEnv H-ala-thr-pro-ser-trp-asn-met-met-met-asp-
 5 trp-ala-met-arg-his-Gly-thr-ile-his-Gly-pro-
 tyr-ile-ser-cys-NH₂

The synthesis of noCEnv was carried out on polyamide
 PepsynKA resin pre-esterified with Fmoc-Ala and riCEnv
 was on Polyhipe Rink resin. The side chain protecting
 10 groups used were: trityl for cysteine, histidine and
 asparagine, t-butyl for serine, threonine and aspartic
 acid and 2,2,5,7,8-pentamethyl chroman-6-sulphonyl for
 arginine. Cleavage and side-chain deprotection were
 accomplished by reaction of the peptidyl resins for 90
 15 min at 0°C with 1M trimethylsilylbromide-thioanisole in
 TFA containing 0.25M 1,2-ethanedithiol (Yajima et al.,
 1988). The peptides were conjugated to BSA as described
 in Example 2.

Both peptides were tested in ELISA using the method
 20 described in Example 11 against sera from HCV positive
 patients in China. NoCEnv detected 15/30 (50%) of
 positive sera tested and riCEnv gave similar results.

EXAMPLE 11

Hepatitis C capsid peptide

25 The following two peptides, based on the capsid
 sequence (39-74) of the hepatitis C virus, were
 synthesised.

noCCap H-Cys-Arg-Arg-Gly-Pro-Arg-Leu-Gly-Val-Arg-
 Ala-Thr-Arg-Lys-Thr-Ser-Glu-Arg-Ser-Gln-Pro-
 30 Arg-Gly-Arg-Arg-Gln-Pro-Ile-Pro-Lys-Val-Arg-
 Arg-Pro-Glu-Gly-Arg-OH (SEQ ID No.9)

riCCap H-Cys-arg-Gly-glu-pro-arg-arg-val-lys-pro-
 ile-pro-gln-arg-arg-Gly-arg-pro-gln-ser-arg-
 glu-ser-thr-lys-arg-thr-ala-arg-val-Gly-leu-
 35 arg-pro-Gly-arg-arg-NH₂

The synthesis of noCCap was carried out on polyamide PepsynKA resin pre-esterified with Fmoc-Arg(Mtr) and riCCap was on Polyhipe Rink resin. The side chain protecting groups used were: trityl for cysteine and
5 glutamine, t-butyl for serine, threonine and glutamic acid, t-butoxycarbonyl for lysine and 2,2,5,7,8-pentamethyl chroman-6-sulphonyl for arginine. Cleavage and side-chain deprotection were accomplished by reaction of the peptidyl resins for 90 min at 0°C with 1M
10 trimethylsilylbromide-thioanisole in TFA containing 0.25M 1,2-ethanedithiol (Yajima et al., 1988). The peptides were conjugated to BSA as described in Example 2.

The wells of PVC microtitre plates were coated with the appropriate antigen (0.05-1µg/well) dissolved in dilute
15 carbonate/bicarbonate buffer pH9.6 and incubated overnight at 4°C. After aspiration, blocking was effected by incubation for 1 h at 37°C with carbonate/bicarbonate buffer pH9.6 containing 20% calf serum. The wells were then washed four times with 0.2%
20 Tween 20 in PBS before addition of antiserum, serially diluted in double strength PBS containing 0.5% BSA, 10% calf serum and 0.2% Triton X 100 (antibody binding buffer). After incubation for 1 h at 37°C, the plates were again washed. Bound IgG was detected by incubation
25 for 30 min at 37°C with suitably diluted affinity purified anti-human IgG horse radish peroxidase in antibody binding buffer. After washing, the plates were developed with 3,3',5,5' tetramethylbenzidine (TMB), 0.01% peroxide at pH5 in the dark. Colour development was
30 stopped by the addition of 2M sulphuric acid and the plates were read at wavelengths of 450nm/630nm.

The BSA conjugated peptides noCCap and riCCap were coated on ELISA plates and tested against sera from Chinese hepatitis C patients. The results are summarised
35 in the table below. In each case cross-reaction between normal and retro-inverso forms of the capsid peptide was observed and with serum No.1 the retro-inverso peptide gave higher titres than the normal peptide.

| Serum Dilution | | | | | | | | | |
|--------------------|-------|----------------|-------|-------|-------|-------|-------|------------------|-------|
| | | Positive Serum | | | | | | Negative Control | |
| Peptide ng/well | Blank | No.1 | | No.2 | | No.3 | | | |
| | | 1:10 | 1:25 | 1:50 | 1:10 | 1:25 | 1:50 | 1:10 | 1:25 |
| noCCap (100ng) | 0.019 | 0.473 | 0.227 | 0.152 | 1.257 | 0.456 | 0.244 | 2.085 | 1.884 |
| riCCap (100ng) | 0.021 | 0.963 | 0.491 | 0.338 | 1.130 | 0.779 | 0.544 | 1.719 | 1.651 |
| noCCap (50ng) | 0.018 | 0.534 | 0.255 | 0.175 | 1.817 | 0.772 | 0.488 | 2.036 | 2.008 |
| riCCap (50ng) | 0.017 | 0.930 | 0.547 | 0.361 | 1.146 | 0.663 | 0.505 | 1.643 | 1.550 |

0.060

0.135

EXAMPLE 12HIV DIAGNOSTIC PEPTIDESPeptides from the envelope protein of the Human immunodeficiency virus

5 The following two peptides, based on residues 579-611 of the envelope protein of the human immunodeficiency virus, were synthesised.

noHIVgp41(579-611) H-Arg-Ile-Leu-Ala-Val-Glu-Arg-Tyr-
 Leu-Lys-Asp-Gln-Gln-Leu-Leu-Gly-Ile-
 10 Trp-Gly-Aba-Ser-Gly-Lys-Leu-Ile-Aba-
 Thr-Thr-Ala-Val-Pro-Trp-Asn-Cys-OH
 (SEQ ID No.10)

riHIVgp41(579-611) H-Cys-asn-trp-pro-val-ala-thr-thr-
 cys(acm)ile-leu-lys-Gly-ser-
 15 cys(Acm)Gly-trp-ile-Gly-leu-leu-gln-
 gln-asp-lys-leu-tyr-arg-glu-val-ala-
 leu-ile-arg-NH₂

 The synthesis of noHIVgp41(579-611) was carried out on polyamide PepsynKA resin pre-esterified with Fmoc-
 20 Cys(trt) and riHIVgp41(579-611) was on Polyhipe Rink resin. The side chain protecting groups used were: trityl for terminal cysteine, glutamine and asparagine, t-butyl for serine, threonine, aspartic acid, glutamic acid and tyrosine, t-butoxycarbonyl for lysine and
 25 2,2,5,7,8-pentamethyl chroman-6-sulphonyl for arginine. Cysteine was replaced by aminobutyric acid (Aba) in noHIVgp41(579-611) and by cysteine with acetamidomethyl (Acm) protection of the sulfhydryl group in
 riHIVgp41(579-611). Cleavage and side-chain deprotection
 30 were accomplished by reaction of the peptidyl resins for 90 min at 0°C with 1M trimethylsilylbromide-thioanisole in TFA containing 0.25M 1,2-ethanedithiol (Yajima et al., 1988). The acetamidomethyl (Acm) protection of cysteine was not removed by this procedure. The peptides were
 35 conjugated to BSA as described in Example 2.

EXAMPLE 13Peptides from gp41 of the Human immunodeficiency virus

The following two peptides, based on the gp41 protein sequence 735-752 of the human immunodeficiency virus, were synthesised.

noHIVgp41(735-753) H-Tyr-Asp-Arg-Pro-Glu-Gly-Ile-Glu-Glu-Glu-Gly-Gly-Glu-Arg-Asp-Arg-Asp-Arg-Ser-NH₂ (SEQ ID No.6)

riHIVgp41(735-753) H-ser-arg-asp-arg-asp-arg-glu-Gly-Gly-glu-glu-glu-ile-Gly-glu-pro-arg-asp-tyr-NH₂

The synthesis of both peptides was carried out on Polyhipe Rink resin. The side chain protecting groups used were: t-butyl for serine, aspartic acid, glutamic acid and tyrosine, and 2,2,5,7,8-pentamethyl chroman-6-sulphonyl for arginine. Cleavage and side-chain deprotection were accomplished by reaction of the peptidyl resins for 90 min at 0°C with 1M trimethylsilylbromide-thioanisole in TFA containing 0.25M 1,2-ethanedithiol (Yajima et al., 1988). A sulfhydryl group was introduced at the N-terminus of these peptides by reaction with 2-iminothiolane.HCl (Traut's Reagent). (Jue, R. et al., 1978). The peptides were conjugated to BSA as described in Example 2.

The wells of PVC microtitre plates were coated with the appropriate antigen (0.05-1µg/well) dissolved in dilute carbonate/bicarbonate buffer pH9.6 and incubated overnight at 4°C. After aspiration, blocking was effected by incubation for 1 h at 37°C with carbonate/bicarbonate buffer pH9.6 containing 20% calf serum. The wells were then washed four times with 0.2% Tween 20 in PBS before addition of antiserum, serially diluted in double strength PBS containing 0.5% BSA, 10% calf serum and 0.2% Triton X 100 (antibody binding buffer). After incubation for 1 h at 37°C, the plates were again washed. Bound IgG was detected by incubation for 30 min at 37°C with suitably diluted affinity

purified anti-human IgG horse radish peroxidase in antibody binding buffer. After washing, the plates were developed with 3,3',5,5' tetramethylbenzidine (TMB), 0.01% peroxide at pH5 in the dark. Colour development was
5 stopped by the addition of 2M sulphuric acid and the plates were read at wavelengths of 450nm/630nm.

The BSA conjugated peptides noHIVgp41(579-611), riHIVgp41(579-611) from Example 12 and noHIVgp41(735-753) and riHIVgp41(735-753) from this Example were coated on
10 ELISA plates and tested in China against sera from patients with HIV positive sera. The results are summarised in the table below. In each case cross-reaction between normal and retro-inverso forms of the gp41 peptide was observed.

| Serum Dilution | | | | | | | | | |
|-----------------------------------|-------|----------------|-------|-------|-------|-------|-------|---------------------|-------|
| Peptide ng/well | Blank | Positive Serum | | | | | | Negative Control | |
| | | No.1 | | No.2 | | No.3 | | 1:50 | |
| | | 1:10 | 1:25 | 1:10 | 1:25 | 1:10 | 1:25 | 1:10 | 1:25 |
| noHIVgp41 (735-753) (200ng) | 0.023 | 0.497 | 0.242 | 0.139 | 0.153 | 0.275 | 0.151 | 0.099 | 0.153 |
| riHIVgp41 (735-753) (200ng) | 0.025 | 0.477 | 0.219 | 0.135 | 0.570 | 0.262 | 0.155 | 0.090 | 0.132 |
| noHIVgp41 (579-611) (200ng) | 0.022 | 0.483 | 0.210 | 0.133 | 0.205 | 0.278 | 0.182 | 0.115 | 0.148 |
| riHIVgp41 (579-611) (200ng) | 0.018 | 0.453 | 0.231 | 0.150 | 0.194 | 0.241 | 0.140 | 0.087 | 0.155 |

EXAMPLE 14

5 Analysis of antibody production and activity of
antibodies raised to peptide sequences corresponding to
residues 126-137 of the surface protein of the Hepatitis
B virus.

The following two peptides, based on residues 126-137 of the surface protein of the Hepatitis B virus, were synthesised.

| | |
|---------------------|---|
| 10 noHBV-S(126-137) | H-Cys-Lys-Thr-Thr-Pro-Ala-Gln-Gly-Asn-Ser-Met-Tyr-Pro-Ser-OH (SEQ ID No.11) |
| riHBV-S(126-137) | H-Lys-ser-pro-tyr-met-ser-asn-Gly-gln-ala-pro-thr-thr-Cys-OH |

15 The synthesis of noHBV-S(126-137) was carried out on polyamide PepsynKA resin pre-esterified with Fmoc-Ser(tBu) and riHBV-S(126-137) was on polyamide PepsynKA resin pre-esterified with Fmoc-Cys(trt). Lysine was not part of the HBV sequence but was included to increase solubility. The side chain protecting groups used were:
20 trityl for cysteine, glutamine and asparagine, t-butyl for serine, threonine and tyrosine and t-butoxycarbonyl for lysine. Cleavage and side-chain deprotection were accomplished by reaction of the peptidyl resins for 90 min at room temperature with 10ml of TFA, 0.25ml 1,2-ethanedithiol, 0.5ml thioanisole, 0.5ml water and 0.75g
25 phenol.

noHBV-S(126-137) was conjugated to KLH and both peptides were independently conjugated to BSA.

30 The KLH conjugate was emulsified in Freund's complete adjuvant (1:1) and used to immunise white Swiss mice according to the following schedule:

- Day 0: 20 μ g of peptide in 100 μ l emulsion, sub-cutaneous (complete Freund's).
- Day 10: 20 μ g of peptide in 100 μ l emulsion, sub-cutaneous (incomplete Freund's).
- 5 Day 20: 20 μ g of peptide in 100 μ l emulsion, intra-peritoneally (incomplete Freund's).

The mice (4 in each group) were bled retro-orbitally five days after the last injection and the serum used in ELISA using microtitre plates coated with 1 μ g/well of the appropriate peptide conjugated to BSA.

10

| | | O.D. 492 nm | |
|----------------|----------|------------------|------------------|
| Plate coating: | | noHBV-S(126-137) | riHBV-S(126-137) |
| Antiserum to: | | noHBV | noHBV |
| Dilution: | | | |
| 15 | 1:50 | 2.551 | 3.057 |
| | 1:100 | 2.673 | 2.946 |
| | 1:200 | 2.317 | 2.876 |
| | 1:400 | 2.161 | 2.355 |
| | 1:800 | 2.039 | 1.746 |
| | 1:1,600 | 1.540 | 1.081 |
| 20 | 1:3,200 | 0.965 | 0.673 |
| | 1:6,400 | 0.619 | 0.481 |
| | 1:12,800 | 0.506 | 0.340 |
| | 1:25,600 | 0.299 | 0.244 |

These results are presented in graph form in Figure 9.

25

EXAMPLE 15

Analysis of antibody production and activity of antibodies raised to peptide sequences corresponding to residues 126-137 of the surface protein of the Hepatitis B virus.

30

The following peptide, based on residues 126-137 of the surface protein of the Hepatitis B virus, was synthesised.

- 40 -

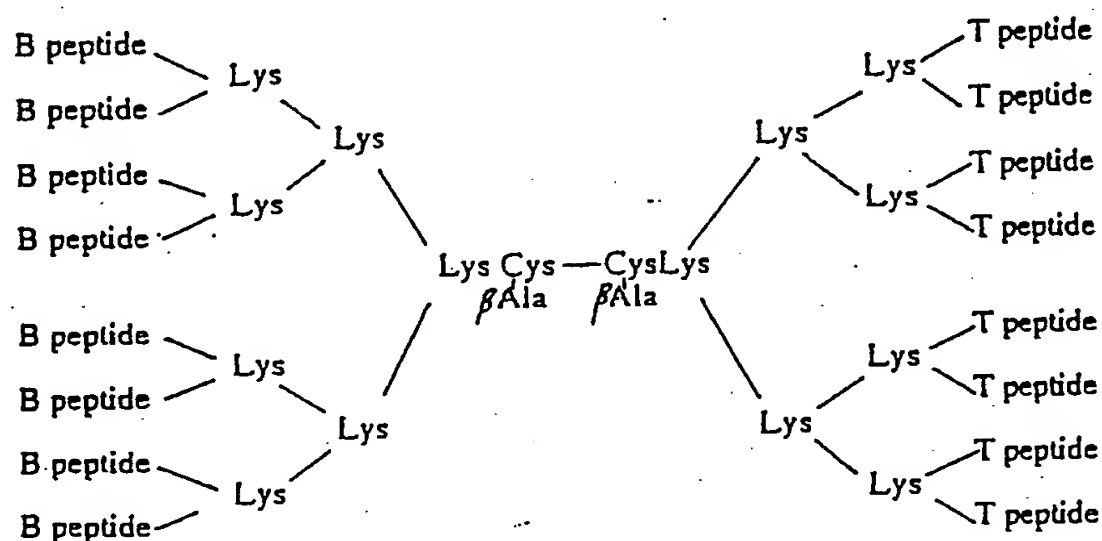
noHBV-S(126-137) H-Thr-Thr-Pro-Ala-Gln-Gly-Asn-Ser-
Met-Tyr-Pro-Ser-OH (SEQ ID No.12)

A potential T cell epitope was selected from the surface protein, residues 20-33:

5 H-Phe-Leu-Leu-Thr-Arg-Ile-Leu-Thr-
Ile-Pro-Gln-Ser-Leu-Asp-OH (SEQ ID
No.13)

These peptides were synthesised on MAP (multiple antigen peptide) resin with eight peptide branches through lysine to each MAP core which was coupled to a cysteine protected by the acetamido methyl group. The side chain protecting groups used were: trityl for glutamine and asparagine, t-butyl for serine, threonine, aspartic acid and tyrosine and 2,2,5,7,8-pentamethyl chroman-6-sulphonyl for arginine. Cleavage and side-chain deprotection of noHBV-S(126-137) were accomplished by reaction of the peptidyl resin for 90 min at room temperature with 10ml of TFA, 0.25ml 1,2-ethanedithiol, 0.5ml thioanisole, 0.5ml water and 0.75g phenol. Cleavage and side-chain deprotection of the potential T cell epitope were accomplished by reaction of the peptidyl resin for 90 min at 0°C with 1M trimethylsilylbromide-thioanisole in TFA, containing 0.25M 1,2-ethanedithiol (Yajima et al., 1988).

25 Dimerisation of the two MAPs, noHBV-S(126-137) and the potential T cell epitope, in equimolar amounts by oxidation to the disulphide with iodine in acetic acid was carried out by the method of Tam and Lu (1989).



The above construct was emulsified in Freund's complete adjuvant (1:1) and used to immunise white Swiss mice according to the following schedule:

5

| | |
|---------|--|
| Day 0: | 20 μ g of peptide in 100 μ l emulsion, sub-cutaneous (complete Freund's). |
| Day 10: | 20 μ g of peptide in 100 μ l emulsion, sub-cutaneous (incomplete Freund's). |
| Day 20: | 20 μ g of peptide in 100 μ l emulsion, intra-peritoneally (incomplete Freund's). |

10

The mice (4 in each group) were bled retro-orbitally five days after the last injection and the serum used in ELISA using microtitre plates coated with 1 μ g/well of either no-HBV-S(126-137) or ri-HBV-S(126-137) both of them conjugated to BSA.

15

20

25

| | | O.D. 492 nm | |
|----------------|--|------------------|------------------|
| Plate coating: | | noHBV-S(126-137) | riHBV-S(126-137) |
| Dilution: | | | |
| 1:50 | | 2.643 | 2.813 |
| 1:100 | | 2.697 | 2.688 |
| 1:200 | | 2.528 | 2.293 |
| 1:400 | | 1.578 | 1.498 |
| 1:800 | | 0.929 | 0.954 |
| 1:1,600 | | 0.548 | 0.571 |
| 1:3,200 | | 0.374 | 0.387 |
| 1:6,400 | | 0.268 | 0.297 |
| 1:12,800 | | 0.248 | 0.269 |
| 1:25,600 | | 0.198 | 0.249 |

These results are shown in graph form in Figure 10.

EXAMPLE 16

5 Analysis of antibody production and activity of
antibodies raised to peptide sequences corresponding to
residues 127-140 of the PreS protein of the Hepatitis B
virus.

The following two peptides, based on residues 127-140 of the preS protein of the Hepatitis B virus, were synthesised.

| | | |
|----|---------------------|--|
| 10 | noHBV-PreS(127-140) | H-Phe-His-Gln-Thr-Leu-Gln-Asp-Pro-Arg-Val-Arg-Gly-Leu-Tyr-Cys-OH (SEQ ID No.14) |
| | riHBV-PreS(127-140) | H-Cys-tyr-leu-Gly-arg-val-arg-pro-asp-gln-leu-thr-gln-his-phe-NH ₂ |

15 The synthesis of noHBV-PreS(127-140) was carried out on polyamide PepsynKA resin pre-esterified with Fmoc-Cys(trt) and riHBV-PreS(127-140) was on Polyhipe Rink resin. The side chain protecting groups used were: trityl for cysteine, histidine and glutamine, t-butyl for threonine, aspartic acid and tyrosine and 2,2,5,7,8-
20 pentamethyl chroman-6-sulphonyl for arginine. Cleavage and side-chain deprotection were accomplished by reaction of the peptidyl resins for 90 min at 0°C with 1M trimethylsilylbromide-thioanisole in TFA containing 0.25M 1,2-ethanedithiol (Yajima et al., 1988).

25 Both peptides were independently conjugated to either KLH or BSA.

The KLH conjugates were emulsified in Freund's complete adjuvant (1:1) and used to immunise white Swiss mice according to the following schedule:

| | | |
|----|---------|--|
| 30 | Day 0: | 20 µg of peptide in 100 µl emulsion, sub-cutaneous (complete Freund's). |
| | Day 10: | 20 µg of peptide in 100 µl emulsion, sub-cutaneous (incomplete Freund's). |
| 35 | Day 20: | 20 µg of peptide in 100 µl emulsion, intra-peritoneally (incomplete Freund's). |

The mice (4 in each group) were bled retro-orbitally five days after the last injection and the serum used in ELISA using microtitre plates coated with 1 μ g/well of the appropriate peptide conjugated to BSA.

| | | O.D. 492 nm | | | |
|----|----------------|-------------------------|--------|-------------------------|--------|
| 5 | Plate coating: | noHBV-PreS (127-140) | | riHBV-PreS (127-140) | |
| | Antiserum to: | no-HBV | ri-HBV | no-HBV | ri-HBV |
| 10 | Dilution: | | | | |
| | 1:50 | 2.477 | 1.468 | 1.125 | 1.253 |
| | 1:100 | 3.108 | 1.425 | 1.150 | 2.035 |
| | 1:200 | 2.892 | 1.284 | 1.325 | 2.345 |
| | 1:400 | 2.997 | 0.577 | 0.636 | 0.976 |
| | 1:800 | 2.582 | 0.269 | 0.409 | 0.248 |
| | 1:1,600 | 1.458 | 0.301 | 0.271 | 0.336 |
| | 1:3,200 | 0.698 | 0.259 | 0.183 | 0.162 |
| | 1:6,400 | 0.638 | 0.217 | 0.256 | 0.283 |
| | 1:12,800 | 0.412 | 0.221 | 0.293 | 0.225 |
| 15 | 1:25,600 | 0.429 | 0.282 | 0.304 | 0.185 |

These results are presented in graph form in Figure 11.

EXAMPLE 17

20 Analysis of antibody production and activity of antibodies raised to peptide sequences corresponding to residues 127-140 of the preS protein of the Hepatitis B virus.

The following peptide, based on residues 127-140 of the preS protein of the Hepatitis B virus, was synthesised.

noHBV-PreS(127-140) H-Phe-His-Gln-Thr-Leu-Gln-Asp-Pro-Arg-Val-Arg-Gly-Leu-Tyr-OH (SEQ ID No.15)

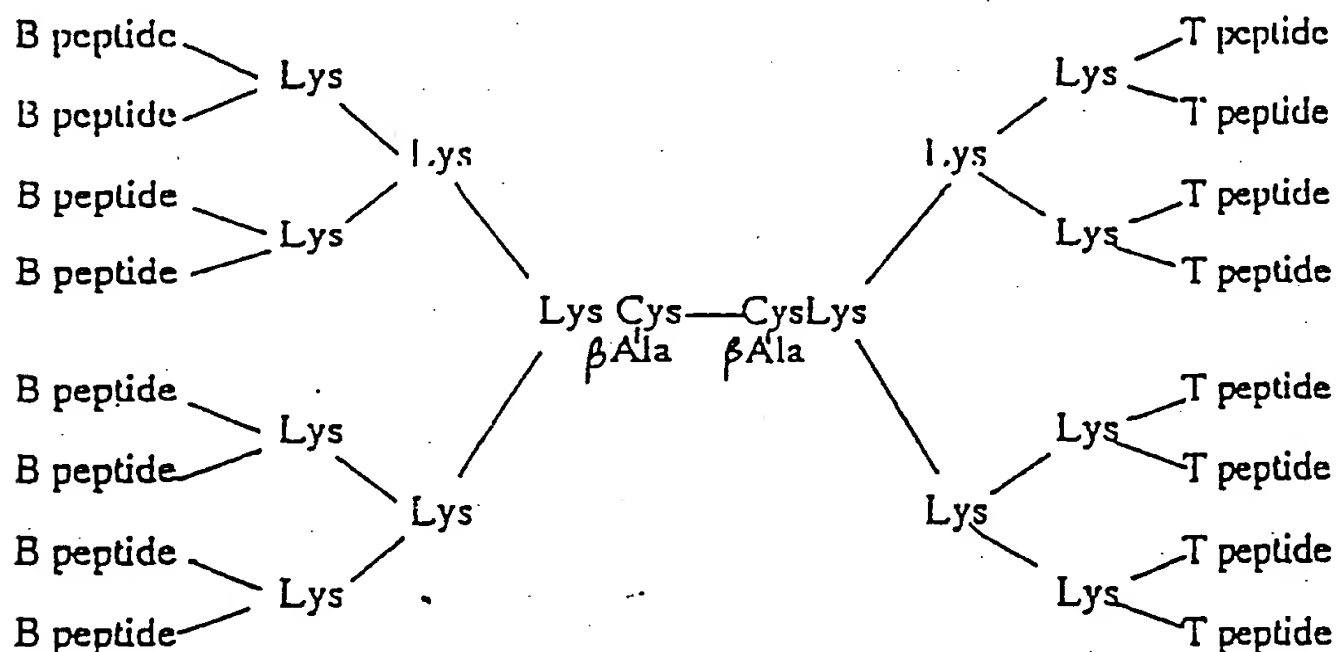
30 A potential T cell epitope was selected from the preS protein, residues 52-64:

- 44 -

H-Trp-Pro-Asp-Ala-Asn-Lys-Val-Gly-
Ala-Gly-Ala-Phe-Gly-OH (SEQ ID
No.16)

These peptides were synthesised on MAP (multiple
5 antigen peptide) resin with eight peptide branches
through lysine to each MAP core which was coupled to a
cysteine protected by the acetamido methyl group. The
side chain protecting groups used were: trityl for
histidine, glutamine and asparagine, t-butyl for
10 threonine and aspartic acid, t-butoxycarbonyl for lysine
and 2,2,5,7,8-pentamethyl chroman-6-sulphonyl for
arginine. Cleavage and side-chain deprotection of noHBV-
PreS(127-140) were accomplished by reaction of the
peptidyl resin for 90 min at 0°C with 1M
15 trimethylsilylbromide-thioanisole in TFA containing 0.25M
1,2-ethanedithiol (Yajima et al., 1988). Cleavage and
side-chain deprotection of the potential T cell epitope
were accomplished by reaction of the peptidyl resin for
90 min at room temperature with 1,2-ethanedithiol (5% by
20 volume) and water (5% by volume) in TFA.

Dimerisation of the two MAPs, noHBV-PreS(127-140) and
the potential T cell epitope, in equimolar amounts, by
oxidation to the disulphide with iodine in acetic acid,
was carried out by the method of Tam and Lu (1989).



The above construct was emulsified in Freund's complete adjuvant (1:1) and used to immunise white Swiss mice according to the following schedule:

5

| | |
|---------|--|
| Day 0: | 20 μ g of peptide in 100 μ l emulsion, sub-cutaneous (complete Freund's). |
| Day 10: | 20 μ g of peptide in 100 μ l emulsion, sub-cutaneous (incomplete Freund's). |
| Day 20: | 20 μ g of peptide in 100 μ l emulsion, intra-peritoneally (incomplete Freund's). |

10 The mice (4 in each group) were bled retro-orbitally five days after the last injection and the serum used in ELISA using microtitre plates coated with 1 μ g/well of either noHBV-PreS(127-140) or riHBV-PreS(127-140) both of them conjugated to BSA.

15

20

25

| O.D. 492 nm | | | | |
|----------------|-------------------------|--------|-------------------------|--------|
| Plate coating: | noHBV-PreS (127-140) | | riHBV-PreS (127-140) | |
| Antiserum to: | no-HBV | ri-HBV | no-HBV | ri-HBV |
| Dilution: | | | | |
| 1:50 | 2.192 | 1.735 | 2.001 | 2.558 |
| 1:100 | 1.900 | 2.111 | 2.121 | 2.457 |
| 1:200 | 2.069 | 2.482 | 1.920 | 2.499 |
| 1:400 | 0.916 | 1.299 | 1.047 | 1.450 |
| 1:800 | 0.325 | 0.454 | 0.685 | 0.751 |
| 1:1,600 | 0.190 | 0.283 | 0.307 | 0.314 |
| 1:3,200 | 0.246 | 0.390 | 0.195 | 0.250 |
| 1:6,400 | 0.275 | 0.224 | 0.261 | 0.244 |
| 1:12,800 | 0.215 | 0.217 | 0.188 | 0.205 |
| 1:25,600 | 0.168 | 0.222 | 0.354 | 0.312 |

These results are presented in graph form in Figure 12.

INDUSTRIAL APPLICATION

Antigen analogues in accordance with the invention have a range of potential applications in eliciting immunogenic responses in a host. These antigen analogues
5 can be used in the treatment and/or prophylaxis of diseases, and drug delivery and therapy of disease states. In particular, these antigen analogues can be used in vaccines in animals, including humans for protection against pathogens and the like.

10 In addition, the synthetic antigen analogues have the potential to be used as diagnostic tools in assays for detecting the presence of antibodies to native antigens or in research such as investigations into antigen-antibody recognition, specificity, antigenicity
15 and immunogenic response.

Antibodies raised against the antigen analogues of the invention can be used for diagnostic purposes where detection of the native antigen is desired in samples or in the treatment and/or prophylaxis of diseases
20 neutralised by the antibodies, or in drug delivery.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: DEAKIN, RESEARCH LTD
COMIS, ALFIO
FISCHER, PETER
TYLER, MARGARET I

(ii) TITLE OF INVENTION: VACCINES

(iii) NUMBER OF SEQUENCES: 16

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: GRIFFITH HACK & CO
(B) STREET: LEVEL 8 168 WALKER STREET
(C) CITY: NORTH SYDNEY
(D) STATE: NEW SOUTH WALES
(E) COUNTRY: AUSTRALIA
(F) ZIP: 2060

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Vers. #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: KURTS, ANN D
(B) REGISTRATION NUMBER: N/A
(C) REFERENCE/DOCKET NUMBER: 21192I:ADK/MT

- 53 -

(ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: 61 2 957 5944
- (B) TELEFAX: 61 2 957 6288
- (C) TELEX: 26547

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

(x) PUBLICATION INFORMATION:

- (H) DOCUMENT NUMBER: AU PL4374
- (I) FILING DATE: 27-AUG-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Gly | Cys | Gly | Pro | Leu | Ala | Gln | Pro | Leu | Ala | Gln | Gly |
| 1 | | | | 5 | | | | | 10 | | |

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: YES

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(x) PUBLICATION INFORMATION:

(H) DOCUMENT NUMBER: AU PL4374

(I) FILING DATE: 27-AUG-1992

(xi) SEQUENCE DESCRIPTION: SEQ. ID NO:2:

Gly Gln Ala Leu Pro Gln Ala Leu Pro Gly Cys Gly
1 5 10

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 13 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Plasmodium falciparum

(D) DEVELOPMENTAL STAGE: SPOROZOITE

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 1..2

(D) OTHER INFORMATION: /label= A

```
/note= "RESIDUE 1 IS AN EXTRA CYSTEINE  
RESIDUE ADDED TO THE N-TERMINUS OF THE  
PEPTIDE"
```


(I) FILING DATE: 27-AUG-1992

Cys Asn Ala Asn Pro Asn Ala Asn Pro Asn Ala Asn Pro
1 5 10

(D) TOPOLOGY: linear

(D) DEVELOPMENTAL STAGE: SPOROZOITE

(I) FILING DATE: 27-AUG-1992

Asn Ala Asn Pro Asn Ala Asn Pro Asn Ala Asn Pro
1 5 10

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(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Corynebacterium diphtheriae*

(x) PUBLICATION INFORMATION:

(H) DOCUMENT NUMBER: AU PL4374

(I) FILING DATE: 27-AUG-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

| | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Gly | Asn | Arg | Val | Arg | Arg | Ser | Val | Gly | Ser | Ser | Leu | Lys | Cys |
| 1 | | | | 5 | | | | | | 10 | | | |

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Human immunodeficiency virus type 1

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 18..19

(D) OTHER INFORMATION: /label= A

/note= "THE C TERMINAL AMINO ACID OF THIS PEPTIDE HAS BEEN AMIDATED. THE ENTIRE PEPTIDE IS BASED ON AMINO ACIDS 735-753 OF THE PROTEIN gp41 OF HIV1."

(x) PUBLICATION INFORMATION:

(H) DOCUMENT NUMBER: AU PL4374

(I) FILING DATE: 27-AUG-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Tyr Asp Arg Pro Glu Gly Ile Glu Glu Glu Gly Gly Glu Arg
1 5 10
Asp Arg Asp Arg Ser
15

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

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(iv) ANTI-SENSE: NO

```
(v) FRAGMENT TYPE: internal
```

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Human immunodeficiency virus type 1

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 16.:17

(D) OTHER INFORMATION: /label= B

```
/note= "THE C TERMINAL RESIDUE OF THIS  
PEPTIDE IS AMIDATED. THE ENTIRE PEPTIDE IS  
BASED ON AMINO ACIDS 583-599 OF PROTEIN gp41  
OF HIV1"
```

(x) PUBLICATION INFORMATION:

(H) DOCUMENT NUMBER: AU PL4374

(I) FILING DATE: 27-AUG-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Leu Gln Ala Arg Ile Leu Ala Val Glu Arg Tyr Leu Lys
1 5 10
Asp Gln Gln Leu
15

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

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(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: HEPATITIS C VIRUS

(ix) FEATURE:

(A) NAME/KEY: Region

(B) LOCATION: 1..25

(D) OTHER INFORMATION: /label= A

/note= "THIS PEPTIDE CORRESPONDS TO AMINO
ACIDS 306-330 OF THE ENVELOPE PROTEIN FROM
HCV."

(x) PUBLICATION INFORMATION:

(H) DOCUMENT NUMBER: AU PL4374

(I) FILING DATE: 27-AUG-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

| | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Cys | Ser | Ile | Tyr | Pro | Gly | His | Ile | Thr | Gly | His | Arg | Met |
| 1 | | | | 5 | | | | | 10 | | | |
| Ala | Trp | Asp | Met | Met | Met | Asn | Trp | Ser | Pro | Thr | Ala | |
| | 15 | | | | | 20 | | | | | 25 | |

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 37 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

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(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: HEPATITIS C VIRUS

(ix) FEATURE:

(A) NAME/KEY: Region

(B) LOCATION: 1..37

(D) OTHER INFORMATION: /label= A

/note= "THIS PEPTIDE CORRESPONDS TO AMINO
ACIDS 39-74 OF THE CAPSID PROTEIN OF HCV."

(x) PUBLICATION INFORMATION:

(H) DOCUMENT NUMBER: AU PL4374

(I) FILING DATE: 27-AUG-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

| | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Cys | Arg | Arg | Gly | Pro | Arg | Leu | Gly | Val | Arg | Ala | Thr | Arg |
| 1 | | | | 5 | | | | | 10 | | | |
| Lys | Thr | Ser | Glu | Arg | Ser | Gln | Pro | Arg | Gly | Arg | Arg | Gln |
| | 15 | | | | | 20 | | | | | 25 | |
| Pro | Ile | Pro | Lys | Val | Arg | Arg | Pro | Glu | Gly | Arg | | |
| | | | 30 | | | | | | 35 | | | |

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 34 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

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(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Human immunodeficiency virus type 1

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 20..26

(D) OTHER INFORMATION: /label= A

/note= "FOR RESIDUES 20 AND 26 Xaa
REPRESENTS AMINOBUTYRIC ACID. THE ENTIRE
PEPTIDE IS BASED ON RESIDUES 579-611 OF THE
ENVELOPE PROTEIN OF HIV1."

(x) PUBLICATION INFORMATION:

(H) DOCUMENT NUMBER: AU PL4374

(I) FILING DATE: 27-AUG-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

| | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Arg | Ile | Leu | Ala | Val | Glu | Arg | Tyr | Leu | Lys | Asp | Gln | Gln |
| 1 | | | | 5 | | | | | 10 | | | |
| Leu | Leu | Gly | Ile | Trp | Gly | Xaa | Ser | Gly | Lys | Leu | Ile | Xaa |
| | 15 | | | | 20 | | | | | 25 | | |
| Thr | Thr | Ala | Val | Pro | Trp | Asn | Cys | | | | | |
| | | | | 30 | | | | | | | | |

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

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(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Hepatitis B virus

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 1..2

(D) OTHER INFORMATION: /label= A

/note= "THE PEPTIDE IS MODIFIED AT ITS N
TERMINUS BY RESIDUES CYS-LYS. RESIDUES 3-14
ARE AMINO ACIDS 126-137 OF HBV SURFACE
PROTEIN."

(x) PUBLICATION INFORMATION:

(H) DOCUMENT NUMBER: AU PL4374

(I) FILING DATE: 27-AUG-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

| | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Cys | Lys | Thr | Thr | Pro | Ala | Gln | Gly | Asn | Ser | Met | Tyr | Pro | Ser |
| 1 | | | | 5 | | | | | | 10 | | | |

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

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(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: HEPATITIS B VIRUS

(ix) FEATURE:

(A) NAME/KEY: Region

(B) LOCATION: 1..12

(D) OTHER INFORMATION: /label= A

/note= "THIS PEPTIDE CORRESPONDS TO RESIDUES
126-137 OF HBV SURFACE PROTEIN."

(x) PUBLICATION INFORMATION:

(H) DOCUMENT NUMBER: AU PL4374

(I) FILING DATE: 27-AUG-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

| | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Thr | Thr | Pro | Ala | Gln | Gly | Asn | Ser | Met | Tyr | Pro | Ser |
| 1 | | | | 5 | | | | | 10 | | |

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

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(vi) ORIGINAL SOURCE:

(A) ORGANISM: Hepatitis B virus

(ix) FEATURE:

(A) NAME/KEY: Region

(B) LOCATION: 1..14

(D) OTHER INFORMATION: /label= A

/note= "THIS PEPTIDE CORRESPONDS TO AMINO
ACIDS 20-33 OF THE SURFACE PROTEIN OF HBV."

(x) PUBLICATION INFORMATION:

(H) DOCUMENT NUMBER: AU PL4374

(I) FILING DATE: 27-AUG-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

| | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Phe | Leu | Leu | Thr | Arg | Ile | Leu | Thr | Ile | Pro | Gln | Ser | Leu | Asp |
| 1 | | | | 5 | | | | | 10 | | | | |

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Hepatitis B virus

(ix) FEATURE:

- ```
(A) NAME/KEY: Region
(B) LOCATION: 1..15
(D) OTHER INFORMATION: /label= A
 /note= "THIS PEPTIDE CORRESPONDS TO AMINO
 ACIDS 127-140 OF THE PRE S PROTEIN OF HBV
 MODIFIED BY THE ADDITION OF A CYS RESIDUE TO
 ITS C TERMINUS."
```

(x) PUBLICATION INFORMATION:

- (H) DOCUMENT NUMBER: AU PL4374  
(I) FILING DATE: 27-AUG-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Phe His Gln Thr Leu Gln Asp Pro Arg Val Arg Gly Leu  
1                    5                    10

Tyr Cys  
15

## (2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Hepatitis B virus



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(D) OTHER INFORMATION: /label= A  
/note= "THIS PEPTIDE CORRESPONDS TO AMINO  
ACIDS 52-64 OF THE PRE S PROTEIN OF HBV."

(x) PUBLICATION INFORMATION:

(H) DOCUMENT NUMBER: AU PL4374

(I) FILING DATE: 27-AUG-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

|     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Trp | Pro | Asp | Ala | Asn | Lys | Val | Gly | Ala | Gly | Ala | Pro | Gly |
| 1   |     |     |     | 5   |     |     |     |     | 10  |     |     |     |

CLAIMS

1. A synthetic peptide antigen analogue of a native peptide antigen, which analogue is partially or completely retro modified, with respect to the native antigen.

2. A synthetic peptide antigen analogue of a native peptide antigen, which analogue is partially or completely inverso modified, with respect to the native antigen.

3. A synthetic peptide antigen analogue of a native peptide antigen, which analogue is partially or completely retro-inverso modified, with respect to the native antigen.

4. An antigen analogue according to claim 3, wherein the amino acid residues flanking the retro-inverted sequence are substituted by side-chain-analogous  $\alpha$ -substituted geminal-diaminomethanes and malonates.

5. A synthetic peptide antigen analogue according to any one of claims 1 to 4 when used to immunize an immunocompetent host.

6. An antigen analogue as defined in any one of claims 1 to 5 wherein the native antigen is a naturally occurring polypeptide or antigenic fragment thereof.

7. An antigen analogue as defined in any one of claims 1 to 6 wherein the antigen analogue is an analogue of: the immunodominant epitope of the circumsporozoite coat protein of *P. falciparum* sporozoites; or a diphtheria toxin antigen; or robustoxin; or a hepatitis virus antigen; or an HIV antigen.



8. An antigen analogue according to claim 2 or 3 which is capable of eliciting an immune response which lasts longer than the immune response obtained with the corresponding native antigen.

5 9. A vaccine comprising an antigen analogue according to one of claims 1 to 8 together with a pharmaceutically acceptable carrier, diluent, excipient and/or adjuvant.

10 10. A vaccine comprising an antigen analogue according to any one of claims 1 to 8 when conjugated with a suitable carrier molecule for administration to a host.

15 11. A vaccine comprising an antigen analogue according to claim 3, wherein the analogue is an analogue of an epitope, and the analogue is modified to protect the C and N termini.

12. An antibody raised against an antigen analogue according to any one of claims 1 to 8 or a vaccine according to any one of claims 9 to 11.

20 13. A method of vaccinating a host in need of such treatment which method comprises administering an effective amount of an antigen analogue according to any one of claims 1 to 8 or a vaccine according to any one of claims 9 to 12 to the host.

25 14. A diagnostic kit comprising an antibody according to claim 12 or an antigen analogue according to any one of claims 1 to 8 together with a positive and a negative control standard.

30 15. A method of preparing an antigen analogue of a native peptide antigen, comprising synthesising a

partially or completely retro, inverso or retro-inverso analogue of the native peptide antigen.

16. A method of preparing a vaccine against a native peptide antigen comprising providing a retro, inverso or  
5 retro-inverso analogue of the native peptide antigen and admixing an effective amount of the antigen analogue with a pharmaceutically or veterinarily acceptable carrier, diluent, excipient and/or adjuvant.

17. A method according to claim 15 which method  
10 additionally comprises conjugating the antigen analogue to a suitable carrier molecule.

18. A method of analysing a sample for a native peptide antigen comprising the use of an antibody according to claim 12 which recognises the antigen.

15 19. A method of analysing a sample for an antibody comprising the use of an antigen analogue according to any one of claims 1 to 8 capable of binding the antibody.

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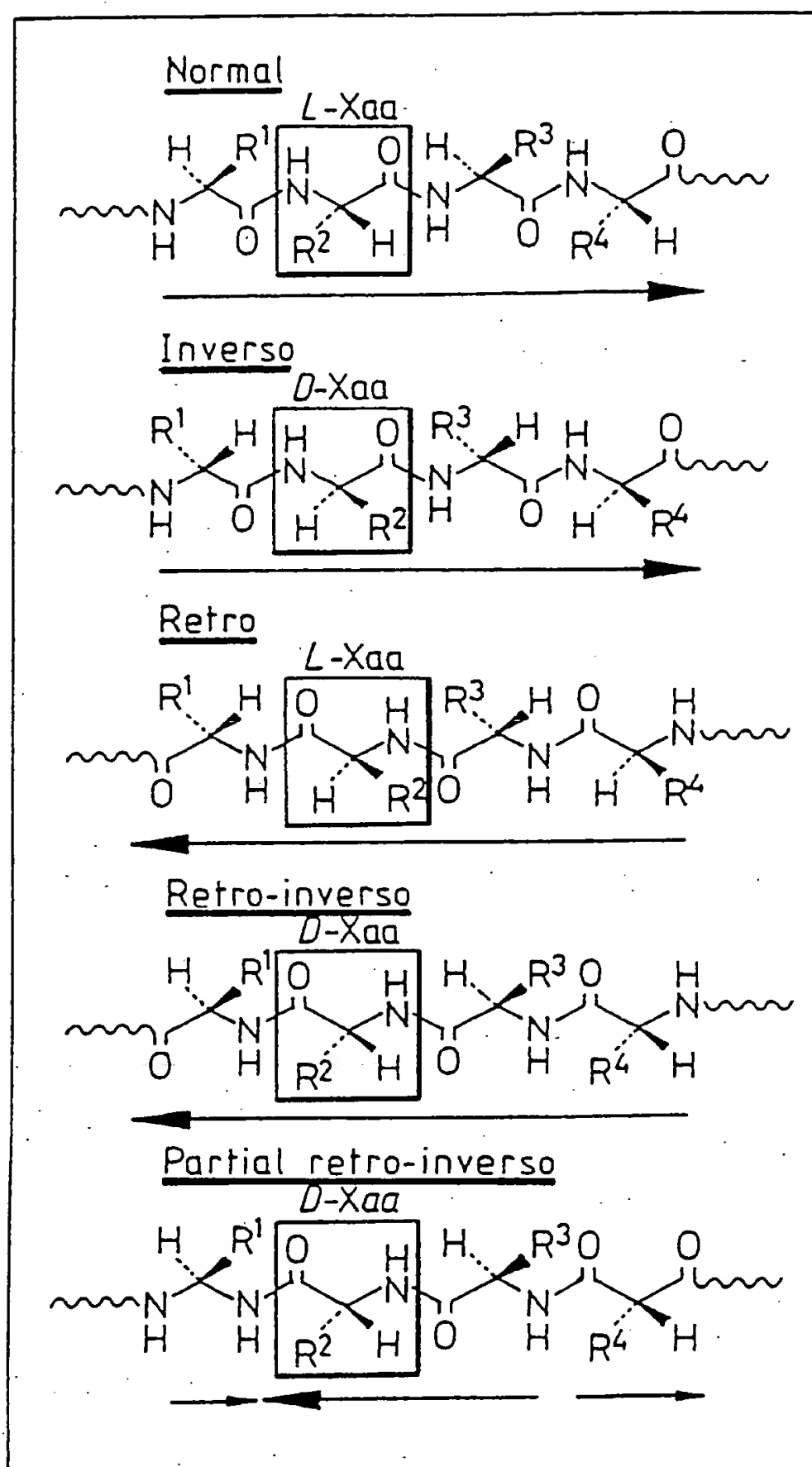


FIG. 1

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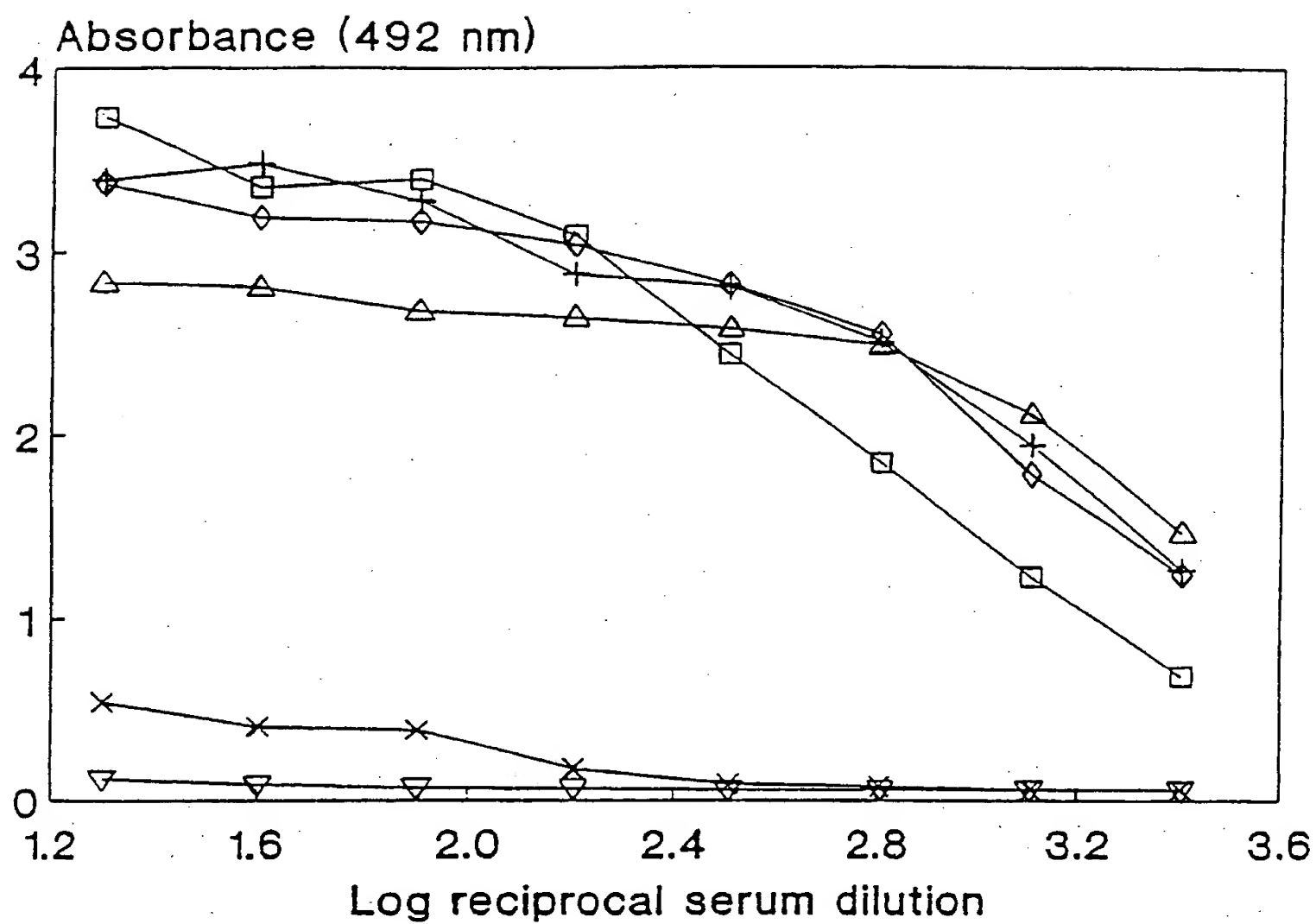


FIG. 2

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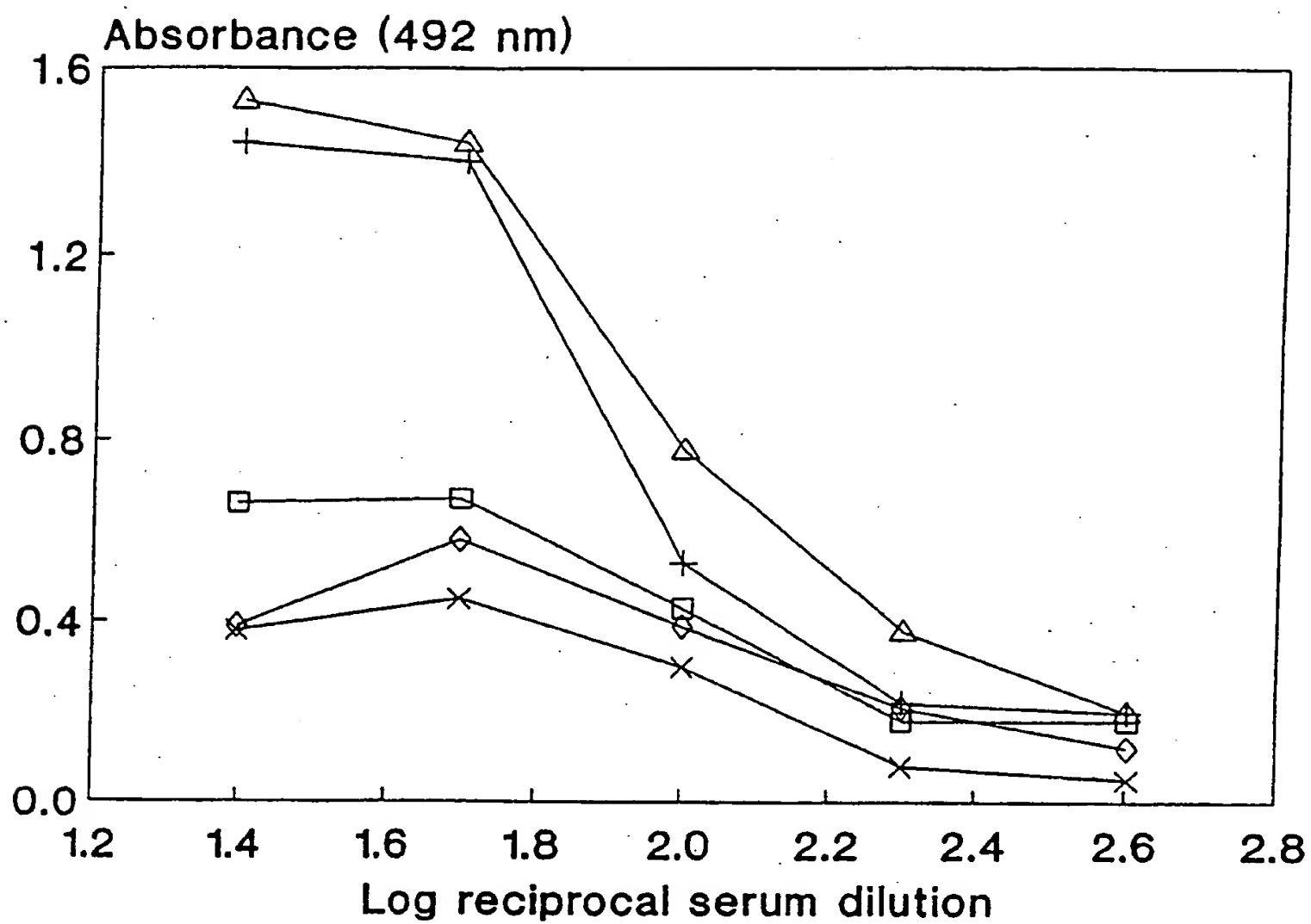


FIG. 3

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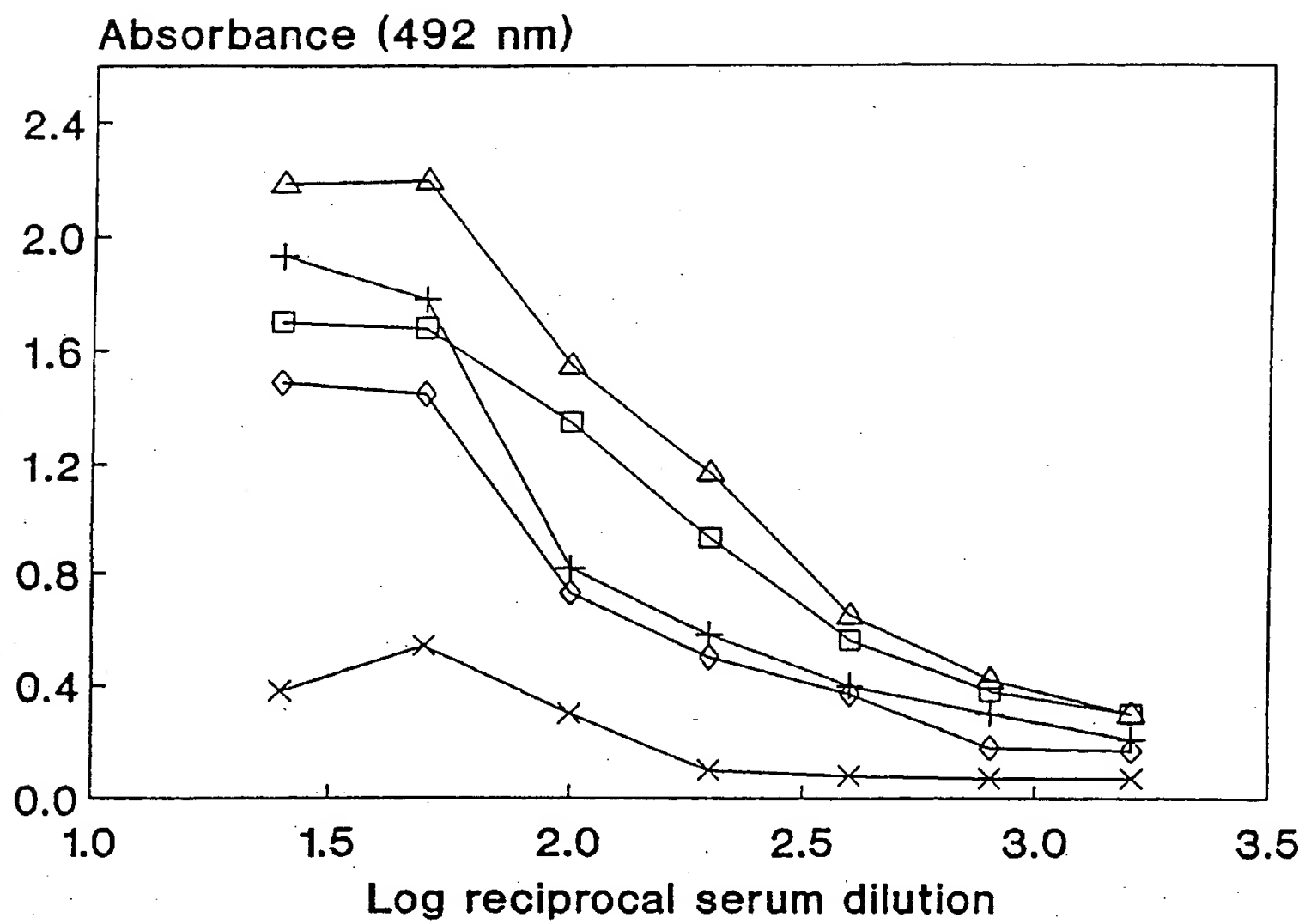


FIG. 4

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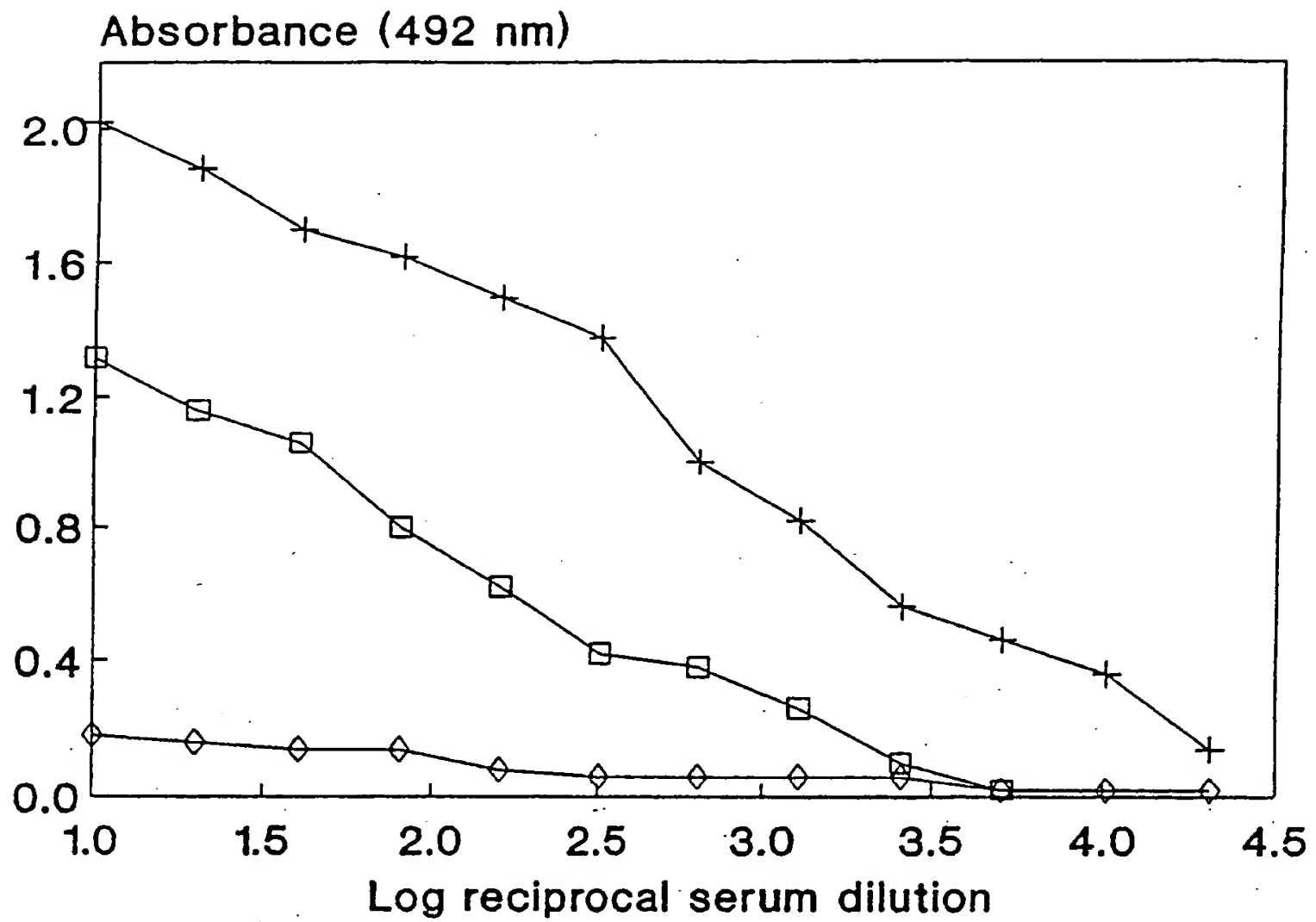


FIG. 5

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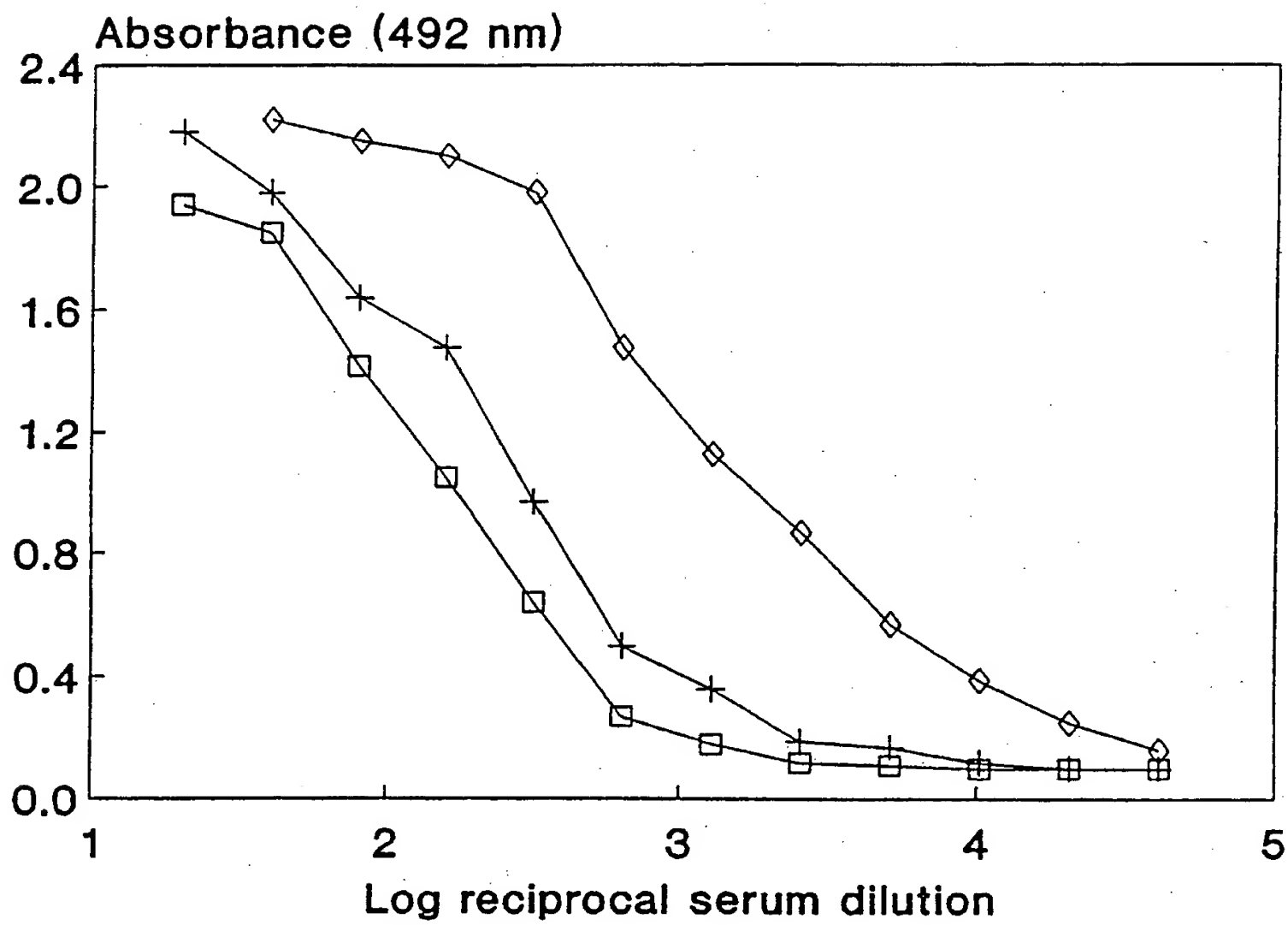
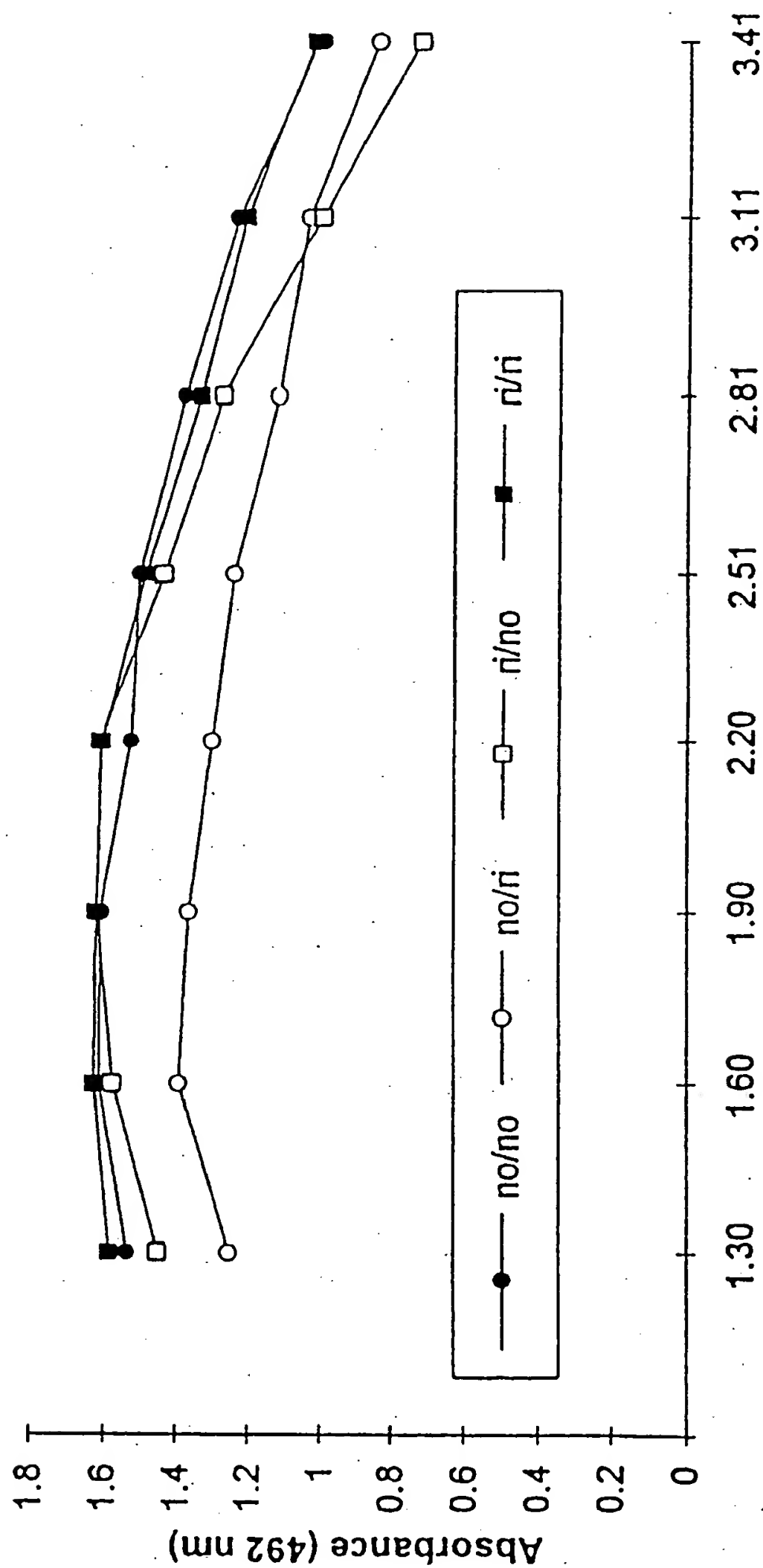


FIG. 6



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Log Recip. Serum Dilution

FIG. 7

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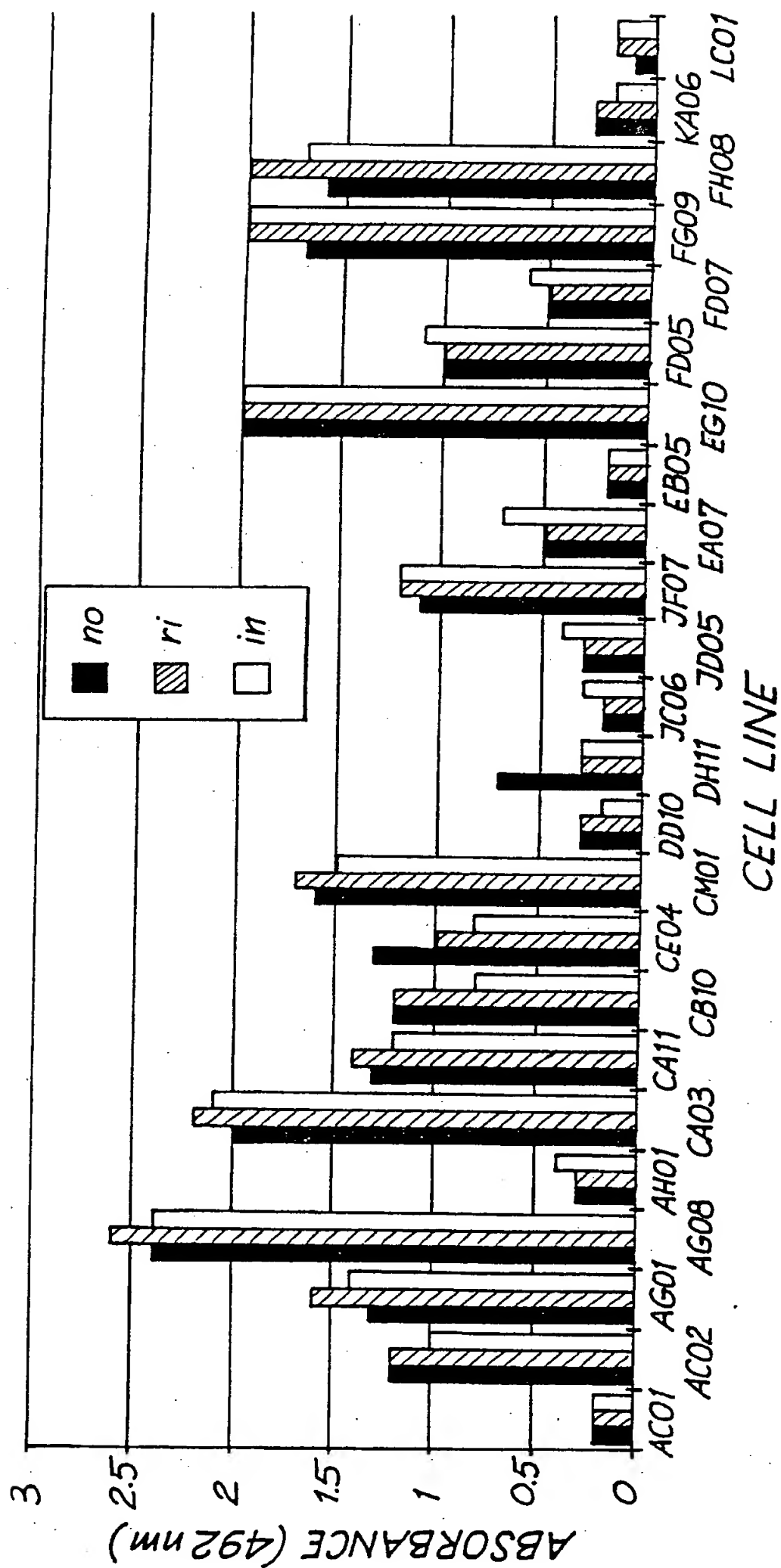


FIG. 8

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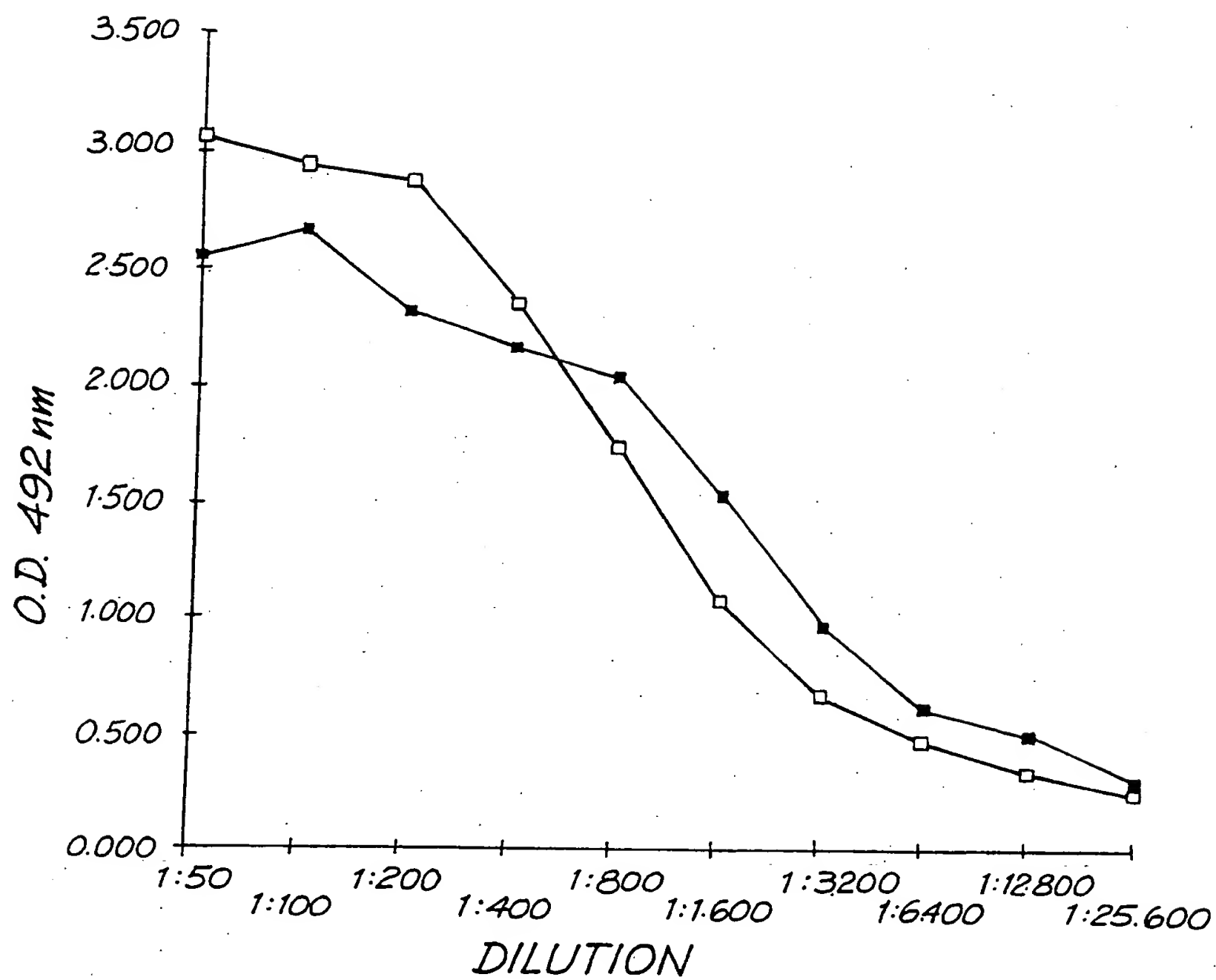
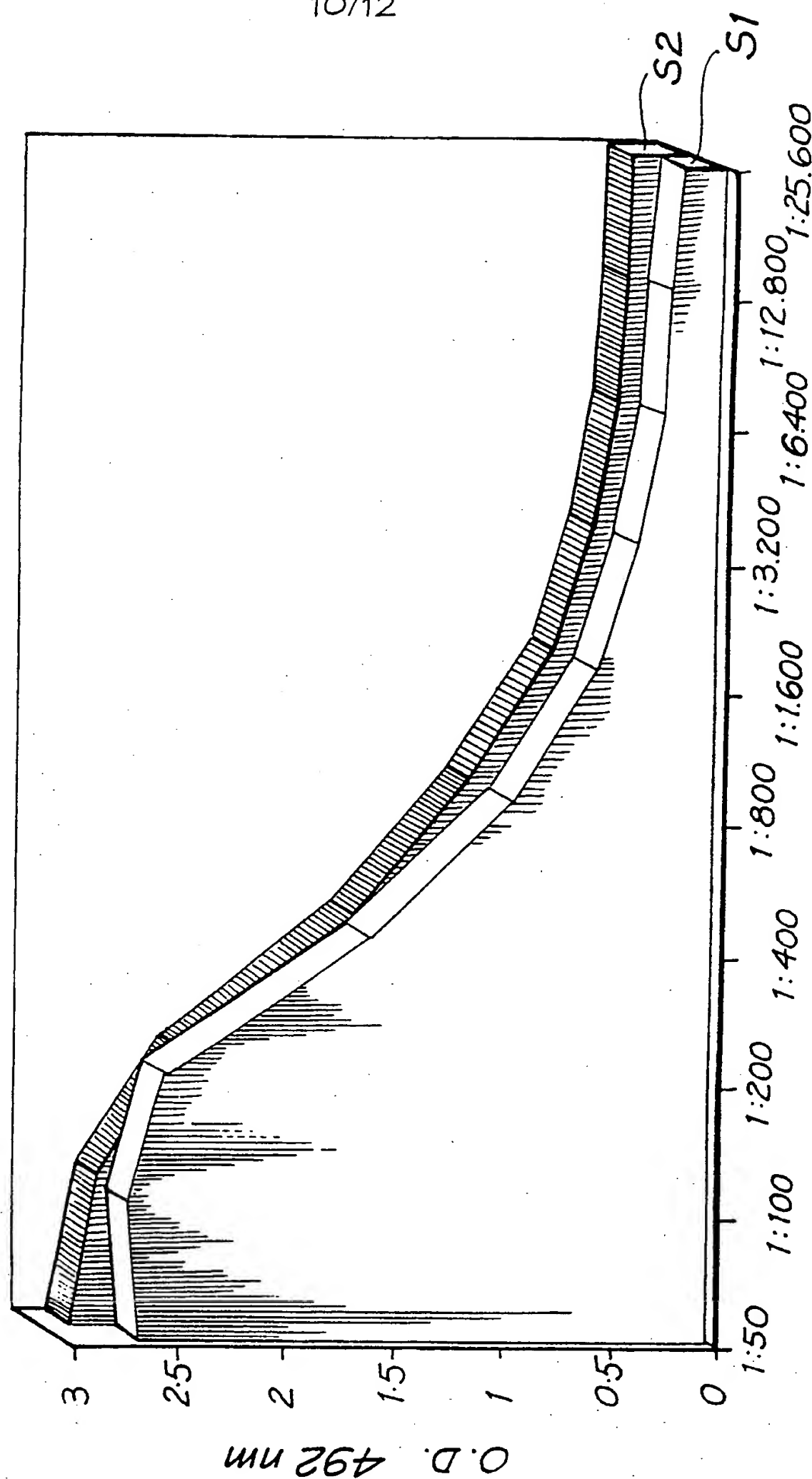


FIG. 9

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DILUTION  
FIG. 10

SUBSTITUTE SHEET

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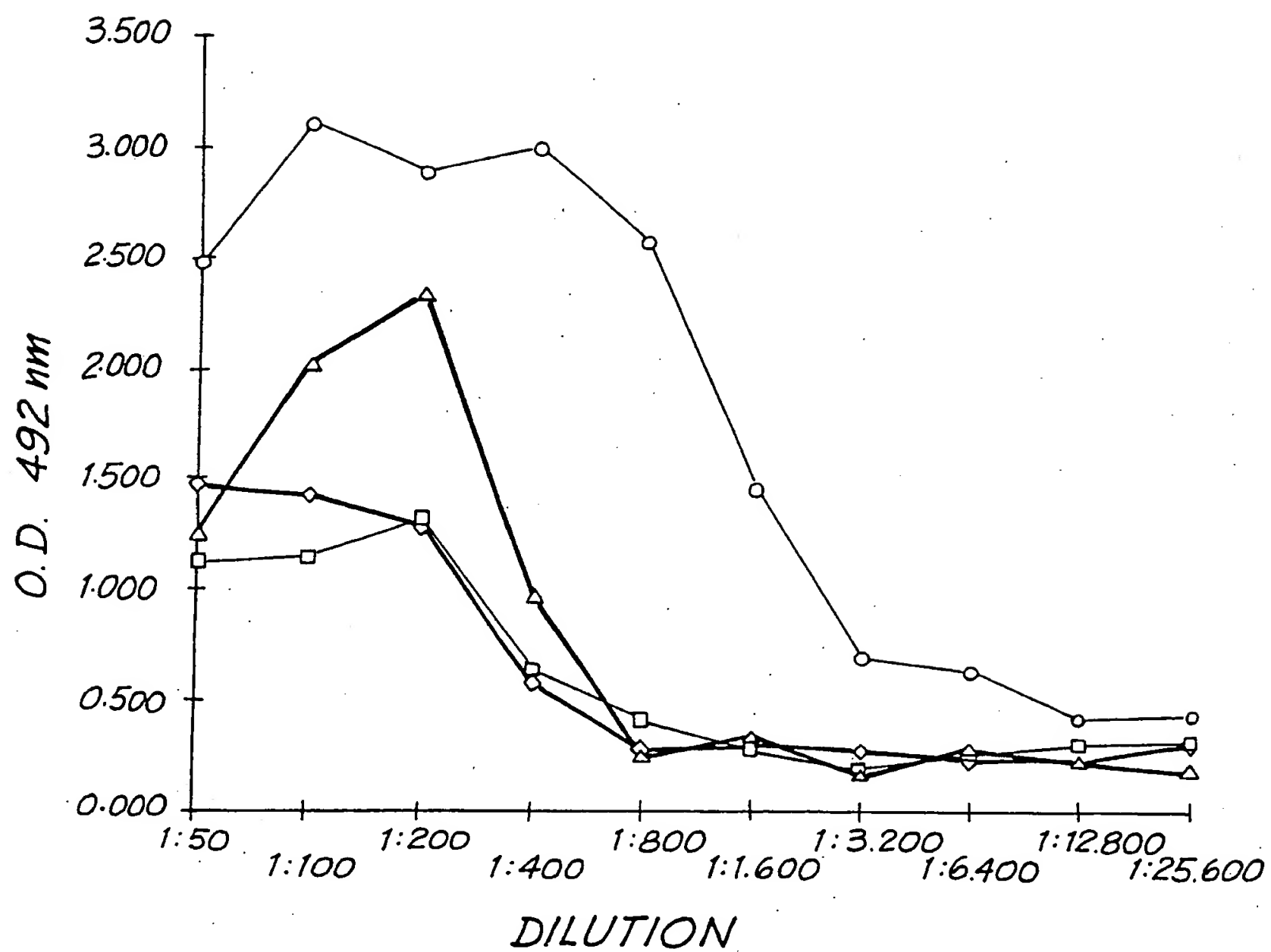


FIG. 11

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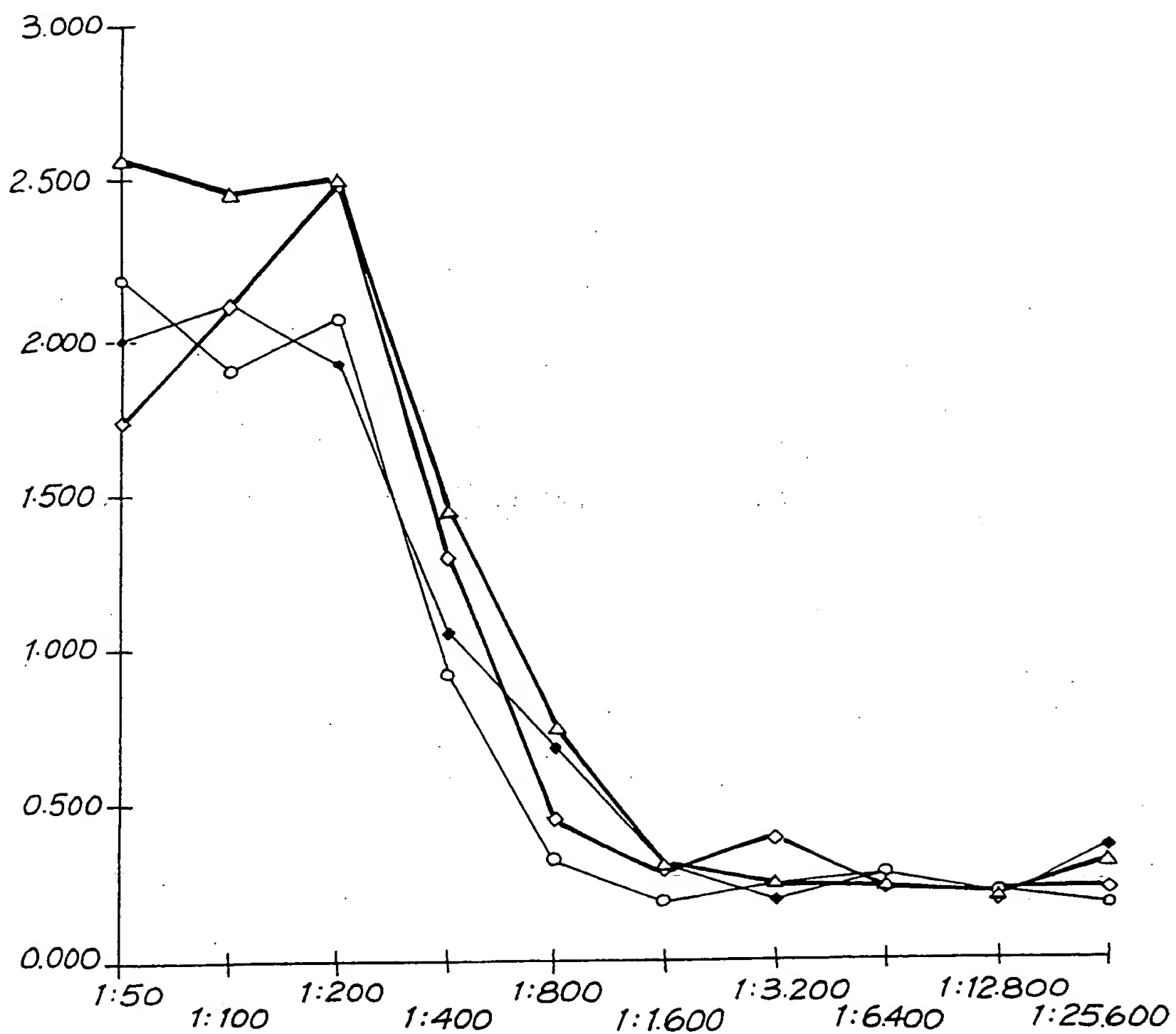


FIG. 12


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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU 93/00441

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| <b>A. CLASSIFICATION OF SUBJECT MATTER</b><br>Int. Cl. <sup>5</sup> A61K 37/02, 39/355, C07K 7/08, 7/10, G01N 33/68<br><br>According to International Patent Classification (IPC) or to both national classification and IPC                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                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| <b>B. FIELDS SEARCHED</b><br><br>Minimum documentation searched (classification system followed by classification symbols)<br>Int Cl <sup>5</sup> C07K, A61K, C07C 103/52<br><br>Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched<br>AU: IPC as above<br><br>Electronic data base consulted during the international search (name of data base, and where practicable, search terms used)<br>DERWENT: RETRO OR INVERSO OR RETRO(W) INVERSO                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        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| <b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  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| Category*                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                 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"The synthesis and confirmational analysis of retro-inverso analogues of biologically active molecules"<br>whole document                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               | 3, 4, 6, 8, 14, 15, 19                                                                                                                                                                                    |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |                                                                                                                                                                          |                                                                                                                                                                                                 |                                                                                                                                                                                                                                              |                                                                                                                                                                          |                                           |                                                                                                                                                                                                                                              |     |                                           |
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"Synthesis and Biological Activity of Some Partially Modified Retro-Inverso Analogues of Cholecystokinin, pages 2331-2339<br>whole document, especially compound 3                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         | 3, 4, 6, 8, 14, 15, 19                                                                                                                                                                                    |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |                                                                                                                                                                          |                                                                                                                                                                                                 |                                                                                                                                                                                                                                              |                                                                                                                                                                          |                                           |                                                                                                                                                                                                                                              |     |                                           |
| <div style="display: flex; justify-content: space-between;"> <div> <input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C.         </div> <div> <input checked="" type="checkbox"/> See patent family annex.         </div> </div>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              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| <table style="width: 100%; border: none;"> <tr> <td style="width: 50%; vertical-align: top;">           * Special categories of cited documents :<br/>           "A" document defining the general state of the art which is not considered to be of particular relevance<br/>           "E" earlier document but published on or after the international filing date<br/>           "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)<br/>           "O" document referring to an oral disclosure, use, exhibition or other means<br/>           "P" document published prior to the international filing date but later than the priority date claimed         </td> <td style="width: 50%; vertical-align: top;"> <table style="border: none;"> <tr> <td style="width: 10%; text-align: center;">"T"</td> <td>later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</td> </tr> <tr> <td style="text-align: center;">"X"</td> <td>document of particular relevance; 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the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone | "Y"                                       | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art | "&" | document member of the same patent family |
| * Special categories of cited documents :<br>"A" document defining the general state of the art which is not considered to be of particular relevance<br>"E" earlier document but published on or after the international filing date<br>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)<br>"O" document referring to an oral disclosure, use, exhibition or other means<br>"P" document published prior to the international filing date but later than the priority date claimed                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 | <table style="border: none;"> <tr> <td style="width: 10%; 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| Date of the actual completion of the international search<br>18 November 1993 (18.11.93)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               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| Name and mailing address of the ISA/AU<br><br>AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION<br>PO BOX 200<br>WODEN ACT 2606<br>AUSTRALIA<br><br>Facsimile No. 06 2853929                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 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                                                                                                                                                                                                                                                                                                                                                                                                                                   |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     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| C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT |                                                                                                                                                                                                                                                                                      |                        |
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| X                                                    | US,A, 4010260 (IMMER, H U et al) 1 March, 1977 (01.03.77)<br>Example 3                                                                                                                                                                                                               | 3, 4, 6, 8, 14, 15, 19 |
| X                                                    | EP,A2, 375040 (SCLAVO SpA) 27 June, 1990 (27.06.90)<br>Example 3                                                                                                                                                                                                                     | 3, 4, 6, 8, 14, 15, 19 |
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# INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU 93/00441

| C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT |                                                                                                                                                                                                                                                                                         |                        |
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| X                                                    | EP,A2, 161007 (ENI-ENTE NAZIONALE IDROCARBURI) 13 November, 1985 (13.11.85)<br>claim 1                                                                                                                                                                                                  | 3, 4, 6, 8, 14, 15, 19 |
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Information on patent family members.

International application No.

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This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

| Patent Document<br>Cited in Search<br>Report |           | Patent Family Member |          |    |          |    |          |
|----------------------------------------------|-----------|----------------------|----------|----|----------|----|----------|
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